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ON THE SPONTANEOUS IMMUNIZATION OF RABBITS TO VACCINE VIRUS

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(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, December 3, 1930)

It has been shown in previous publications (1, 2) that in the testicle, especially, and also to some degree in certain other organs, there exists a factor which is capable of greatly augmenting the lesions produced by viruses and bacteria. In the course of experiments designed to test the possibility of neutralizing this factor by an antitesticular serum, a number of rabbits were given repeated injections of testicle extract subcutaneously, intravenously and intraperitoneally. As a control, others were similarly injected with spleen extract. In the course of testing the effects of the resultant sera on the factor of testicle extract, it was observed that in certain instances not only was the enhancing power of testicle extract neutralized, but the vaccine virus used in the test was inactivated. An examination was made into this unexpected result.

The rabbits comprising the experiment during the immunization period of from 2½ to 3½ months had been kept in individual cages in a room with a number of other rabbits bearing active vaccinal skin lesions. This fact led to a suspicion that the test animals might have acquired an unnoticed vaccine infection. To check this point, all animals in subsequent experiments were subjected to careful scrutiny for possible manifestation of infection. In one instance three or four very small papules were observed, which proved on transfer to be true vaccinia.

The active immunity of all rabbits of these series showing virus neutralizing bodies in their sera was tested by direct inoculation of vaccine virus in the skin. As a control, the protective power

of the serum, and the active immunity of 15 rabbits which had been inoculated with a transplantable rabbit tumor and kept in the same room, were tested. For the purpose of the present discussion, the room in question will be termed the infected room.

The results of this test were as follows. Among the 21 rabbits repeatedly injected with testicle or spleen extract, and repeatedly bled from the ear vein, with an average residence in the infected room of from 2½ to 3½ months, 8 were immune to vaccine virus; 6 among these had received testicle injections and 2 had received injections of spleen extract. Of the 15 rabbits which had been injected only once with tumor tissue and had had two months residence in the infected room, 2 proved to be immune to vaccine virus.

TABLE 1

	NUMBER OF ANIMALS TESTED	NUMBER OF ANIMALS FOUND IMMUNE	PERCENTAGE OF SPONTANEOUS IMMUNIZATIONS
Rabbits living in infected room and repeatedly injected with testicle or spleen extracts......	21	8	38
Rabbits living in infected rooms and injected only once with cancer tissue in testis........	15	2	13
Rabbits living in uninfected room and repeatedly injected with testicle extract.......	14	0	0

As a control to the above observation, a third group of 14 rabbits was repeatedly injected with testicle extract and repeatedly bled in the same manner as the first series, but kept in a room where no animal infected with vaccine virus had ever lived. Tests showed that none of these animals had acquired immunity to vaccine virus. For comparison the results are brought together in table 1.

CONCLUSIONS

The examination of the results shown in table 1 leads to the conclusion that repeated injury from injection and bleeding increased the possibility of spontaneous infection in an infected room. That this possibility was not materially influenced by the

injection of an enhancing factor is evident by the fact that some animals injected with spleen or tumor tissue without enhancing power became immune as well as those receiving testicle extract. The part played by repeated injury is indicated by the low incidence of spontaneous immunization (13 per cent) among the tumor animals which received a single injection, compared with the much higher figure (38 per cent) for the rabbits repeatedly injected with testicle and spleen extracts.

The easy possibility of unnoticed infections leading to development of subsequent immunity (3, 4) has an important bearing on the interpretation of the recently much discussed question of the production of immunity by killed vaccine virus (5).

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STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

VII. OBSERVATIONS ON RABBITS INOCULATED WITH A TRANSPLANTABLE MALIGNANT NEOPLASM

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(Received for publication, March 13, 1931)

Previous papers of this series contain successive observations on the blood count of groups of normal rabbits examined over prolonged periods of time (1, 2, 3, 4, 5). The striking feature brought out by the analysis of these results was the character of the numerical variations shown by the several classes of cells and by the hemoglobin content; it appeared that certain of these variations were of an orderly nature rather than chance occurrences and that in many instances, they appeared to be related to environmental (seasonal) conditions. A further statistical analysis showed, moreover, that a significant association existed between the variations of certain cells while in other cases, no relation whatever was demonstrated (6). The magnitude of the variations, it should be pointed out, was within the limits of what is usually considered normal.

The object of the observations on normal rabbits was not only to obtain information on the character of the blood picture with respect to environmental conditions, but in addition, to provide a background for similar observations in experiments dealing with the reaction of rabbits to various disease agents. The agents employed in these experiments were *Treponema pallidum* (7), virus III disease of rabbits (8), and a transplantable malignant neoplasm (9). In connection with these studies on the blood picture of inoculated rabbits, parallel observations have also been made on tissues involved in the disease process, the idea being that the findings in the one might be related to those of the other and thus open up a method of experimental approach to the question of the nature of the host's reaction. Still other studies have been carried out with respect to the reaction of the

host from the standpoint of the character of the blood picture before inoculation. These two aspects of our work will be reported later.

The present paper contains the results of successive observations on the peripheral blood picture of rabbits inoculated with a transplantable malignant neoplasm. The tumor has been carried in this laboratory for 10 years and has been used in many experiments under diverse circumstances (10); it is considered to be of epithelial origin.

Material and Methods

The material used in this study was derived from observations on 9 groups of rabbits, a total of 78 animals. The dates of inoculation and other data are given in Table I.

TABLE I

Group	No. of rabbits	Tumor inoculation	First blood examination	Last blood examination	Number of blood examinations*	
					Before inoculation	After inoculation
I	10†	Nov. 17, 1927	Oct. 24, 1927	Jan. 18, 1928	4	9
II	5	Jan. 5, 1928	Dec. 12, 1927	Feb. 14, 1928	9	19
III	9	Feb. 24, 1928	Feb. 8, 1928	Apr. 25, 1928	4	9
IV	10†	Apr. 20, 1928	Apr. 6, 1928	June 20, 1928	4	9
V	10	Nov. 22, 1928	Nov. 2, 1928	Jan. 21, 1929	4	8
VI	5†	Jan. 4, 1929	Dec. 29, 1928	Mar. 8, 1929	3	8
VII	12	Nov. 19, 1929	Oct. 29, 1929	Jan. 13, 1930	6	15
VIII	12	Jan. 14, 1930	Dec. 31, 1929	Mar. 18, 1930	5	9
IX	8	Jan. 14, 1930	Dec. 31, 1929	Mar. 18, 1930	5	9
Total....	81				44	95

* In certain series, additional counts prior to the selected preinoculation period as well as the extra counts on a few animals kept 10 weeks after inoculation are not included.

† One rabbit each of Groups I and IV omitted because of a complicating nephritis; one animal of Group VI omitted because it was killed for transfer material before the end of the experiment.

The rabbits were adult male animals approximately 6 to 8 months of age at the time of inoculation. They were representative of the usual stock received from dealers and may be described as brown, black, and Flemish crosses. In the case of Group IX, the rabbits were born and raised in this laboratory; their ages varied from 6 to 12 months and for the most part, they also were of hybrid stock. Dur-

ing the entire observation period, each animal was caged separately; the diet consisted of hay, oats, and cabbage.

The tumor inoculations were carried out with a heavy suspension of an actively growing tumor in sterile normal saline, 0.3 cc. being injected in one testicle. All animals of a group were inoculated at the same time.

The course of the disease was followed in each animal by frequent clinical examinations, special attention being paid first to the time of initiation of the growth of the primary tumor, its general rate of growth, and its ultimate condition, that is, continued growth, regression, or healing; second, the time of appearance, the location, and the course of metastases in superficial parts of the body; and third, the general physical condition including body weight determinations. The experiments were discontinued 8 to 9 weeks after inoculation, at which time all surviving animals were killed by an injection of air into the marginal ear vein. The same procedure was carried out in the case of any animal whose condition became critical before the end of the observation period. A few rabbits developed "snuffles" while under observation, but in the present analysis of results, they have not been separately considered.

The character of the disease was further appraised by the postmortem examination findings of each rabbit. The site of any macroscopic tumor growth was noted and in addition, such features as size, destructiveness, and approximate amount of living and necrotic tumor tissue were described. In the case of those rabbits in which tumor was found at the end of the experimental period, a classification of probable death or probable recovery was made, based upon the location and character of the tumor growths. The probable deaths, for example, include those instances in which both suprarenal glands or the hypophysis were involved by apparently living tumor while the probable recoveries include cases in which only necrotic and evidently healing tumors were found in such locations as the retroperitoneal lymph nodes. This classification is based upon the results of a 10 years' experience with this tumor.

The general conduct of the experiments and the technique employed in the blood examinations were similar to those carried out on normal rabbits (1). Suffice it to say here that a variable number of examinations were made during the 3 weeks preceding inoculation and once a week thereafter during the postinoculation period of 2 months. In the case of Groups II and VII, the examinations were made more frequently; in the present analysis of results, these observations have been averaged for each week. The red and white blood cell counts were made with standardized pipettes; the hemoglobin determinations were carried out with a Newcomer hemoglobinometer. Differential white blood cell counts were made with the supravital neutral red technique, 100 cells being counted in each preparation.

In the present consideration of results, each animal has been allocated to one of the four following groups depending upon the postmorten findings: deaths, probable deaths, probable recoveries, and recoveries. The findings of each weekly blood examination of each individual animal in these groups were calculated in terms of

the percentage deviation from so called standard values (1, 2). These percentage values were then combined in the form of an algebraic sum for each of the four animal groups and mean group values for each week were then calculated. By this method, individual animal and series peculiarities are minimized and in addition, the final figures may be considered as resembling smoothed values.

RESULTS

The results of nine experiments dealing with the peripheral blood cytology in rabbits inoculated with a transplantable malignant neoplasm are presented in a series of curves contained in Text-figs. 1 to 8. Because of space limitations, it has not been possible to include either the figures of individual observations or the combined weekly values. The curves represent in the form of mean percentage deviations from so called standard values, successive week to week levels of the eight blood elements studied, that is, the red blood cells, the hemoglobin content, the total white cells, the neutrophiles (pseudo-eosinophiles), the basophiles, the eosinophiles, the lymphocytes, and the monocytes respectively.[1] Each chart comprises four curves corresponding to the four groups in which the rabbits have been classified, that is, those animals which died as a result of the malignant disease, those which probably would have died had the experiments been continued, those which probably would have recovered, and finally, the recoveries or those rabbits in which no tumor was found at conclusion of the experiments.

As determined by clinical examination, a definite primary tumor developed in each rabbit with six exceptions, that is, one animal each

[1] The standard values used in this analysis are derived from 1110 blood counts on 174 normal rabbits (1, 2) and are as follows:

	per c.mm.
Red blood cells	5,200,000
Hemoglobin	63%
White blood cells	9560
Neutrophiles	4340
Basophiles	950
Eosinophiles	215
Lymphocytes	3050
Monocytes	1000

The curves of the present paper are drawn on the same scale as those which represent the findings in groups of normal rabbits observed for long periods of time and which were analyzed on the basis of the above values (2, 3, 4, 5).

in Groups III, VI, VIII, and IX and two animals in Group VII.[2] Metastases in such locations as the skin, the eyes, and the superficial lymph nodes were observed clinically in several rabbits and there were a number of cases in which paralysis of the hind quarters developed, due to metastatic growths in the spine. In the case of the recoveries and the probable recoveries, an excellent physical condition was maintained. This was also true in certain of the animals classified as probable deaths but not in others, particularly in those cases in which metastatic involvement of the jaws occurred. With respect to those rabbits which died from the disease, the physical condition was affected usually during the week preceding the fatal outcome.

Table II shows the distribution of animal material of the nine experiments; the sole criterion of this distribution, it may be pointed out again, was the character of the disease.

TABLE II

Group	No. of rabbits	Deaths	Probable deaths	Probable recoveries	Recoveries
I	9	5	2	0	2
II	5	2	0	2	1
III	9	3	4	1	1
IV	9	3	1	2	3
V	10	2	1	2	5
VI	4	2	0	1	1
VII	12	5	3	4	0
VIII	12	2	4	5	1
IX	8	1	1	5	1
Total.........	78	25	16	22	15

DISCUSSION

There are certain general features of these experiments which should be referred to before discussing the results.

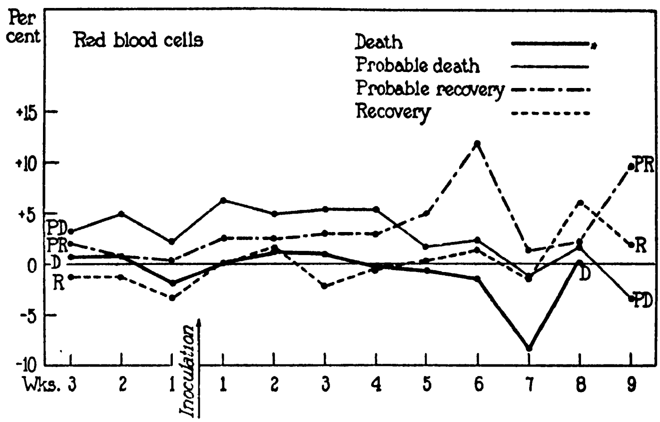
[2] It should be mentioned in this connection that tumor growths in other parts of the body may be found in cases in which a primary tumor fails to develop after intratesticular inoculation. Among the present six rabbits in which the primary tumor did not develop, four showed tumors elsewhere at postmortem examination 2 months after inoculation. The two rabbits in which no tumor was found have been classified as recoveries; there may have been metastatic growths which had regressed and healed.

In the first place, it must be remembered that the experiments were carried out in different years and in different seasons of the same year. It is to be expected, therefore, that some spontaneous variations in the blood picture might occur as in the case of normal rabbits observed over similar periods of time (2, 3, 4, 5). Such variations, which may be attributable to general environmental conditions as well as the occasional irregular values of individual animals, are minimized by the group method employed for the analysis of the results. Secondly, it should be pointed out that the course of this neoplastic disease is subject to considerable variation, not only as regards individual animals of the same series, but in different series as a whole. These variations are shown by such features as the rate of growth of the primary and metastatic tumors and the death rate, the time at which a fatal outcome occurs, the incidence, distribution, and extent of metastases, and the number of recovered animals. In some series, the degree of malignancy is high while in others it is low and in still others, it lies between these extremes. In the present experiments, as shown in Table II, the varying numbers of deaths, of probable deaths, of probable recoveries, and of recoveries indicate the different degrees of malignancy presented by the animals of these experiments. It is evident that the combination of individual animal observations derived from successive weekly examinations is subject to correction on the basis of the course of the disease in each particular animal. It would be difficult to follow this procedure, however, because the only available features of the disease which lend themselves to a practical method of appraisal are, first, the primary tumor, the development and growth of which is not necessarily an index of tumor growth elsewhere, and second, the occurrence of metastases in superficial parts of the body. The relatively low incidence of such superficial tumors except in cases of evident high malignancy, precludes the use of this feature. It has been found by experience, however, that gross changes in tumor growth are usually evident from week to week, and hence this time interval was used for the present observations. The combination of the results of individual animals from different series has been made on the same time basis as affording the least opportunity for error.

In the third place, the results have been considered from the standpoint of the mean values of a group rather than from that of the individual animal since exaggerated or unusual findings in particular rabbits as well as the results of technical errors of blood examination are thereby minimized. While the numbers of rabbits in each group are not large, they are sufficiently comparable to permit group comparisons, that is, 25, 16, 22, and 15 animals in the deaths, the probable deaths, the probable recoveries, and the recoveries, respectively. Finally, it must be remembered in comparing the results of the four groups that in the case of the fatal group, its numbers became progressively decreased and the reliability of the mean values is thereby lessened. 5 weeks after inoculation there were 24 animals; at the 6th week, 17; at the 7th week, 10, while there were but 3 at the 8th week. It should also be remembered with respect to the rabbits in the group of probable recoveries that all showed some evidence of tumor at postmortem

examination. In many instances, the growths appeared to be largely or entirely necrotic and in all cases what tumor there was, either living or necrotic, was in a location which presumably would not have led to the death of the animal; had the experiments continued, it was felt that ultimate regression and healing would have occurred.[3] In six instances, the residual tumor comprised only necrotic nodules in the inoculated testicle.

The results obtained will now be discussed, beginning with the erythrocytes and the hemoglobin content and then taking up the total white count and the various classes of white cells. The most striking changes were observed in the fatal group as might be expected, and therefore, these results will first be considered; the recovered group, on the other hand, showed comparatively slight alterations.



TEXT-FIG. 1. Red blood cells. In this and subsequent charts, the values for the four animal groups are expressed in the form of mean percentage deviations (algebraic sum) from standard values.

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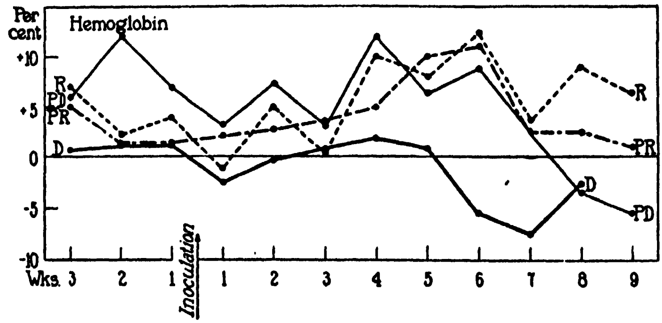
[3] In this connection, it should be mentioned that it has not been possible to reinoculate recovered tumor rabbits by any of the usual routes, such as the intravenous, the intracutaneous, or the intracerebral. The only site which so far has proven successful has been the suprarenal gland (unpublished experiments of Pearce and Van Allen); other tissues appear to be immune (11). It appears extremely unlikely that both suprarenals will eventually become involved when the only tumor present 2 months after inoculation is confined to the residual primary tumor or to growths in such comparatively remote sites as the eye, the connective tissue, or the retroperitoneal lymph nodes. It is also unlikely, even if one suprarenal is the seat of a metastasis, that the second will become involved after 2 months of the disease. All such cases are classified as probable recoveries.

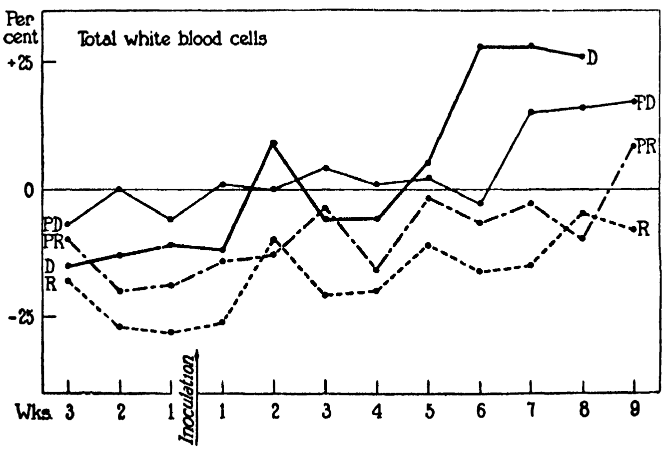


TEXT-FIG. 2. Hemoglobin content

Red Blood Cells and Hemoglobin:—The curves representing the mean levels of the red blood cells during the 3 weeks prior to inoculation (Text-fig. 1) show a range from 4 per cent above the standard value of 5,200,000 cells per c.mm. in the case of the probable deaths, to 3 per cent below this value in the recovered group; the curves representing the deaths and the probable recoveries lie between these limits. For the two first observations after inoculation, the red cell values became increased in all four groups as is shown by the upward trend of the curves. In the case of the fatal group, the curve then describes a gradual fall for 4 weeks, with an abrupt and marked drop to minus 7 per cent at the 7th week and an equally abrupt terminal rise to the base line. It will be remembered that the last two examinations present but 10 and 3 animals; the rise of the last observation was caused by the results on one rabbit, the last values of which, however, were much lower than the preceding ones. The curve of the probable deaths is maintained at approximately a plus 5 per cent level through the 4th week; it then descends in a fairly orderly fashion to a final level of 3 per cent below the base line. The findings in the probable recoveries and the recoveries were more irregular than those of the other groups, but there was a definite tendency toward progressively higher values, particularly in the last four observations; the curves for both groups end well above the base line.

The mean values of the hemoglobin content were, with the exception of the fatal group, more irregular than those of the red blood cells. This feature is brought out by comparing the curves of Text-figs. 1 and 2; it will be seen at once that in the curves representing the hemo-

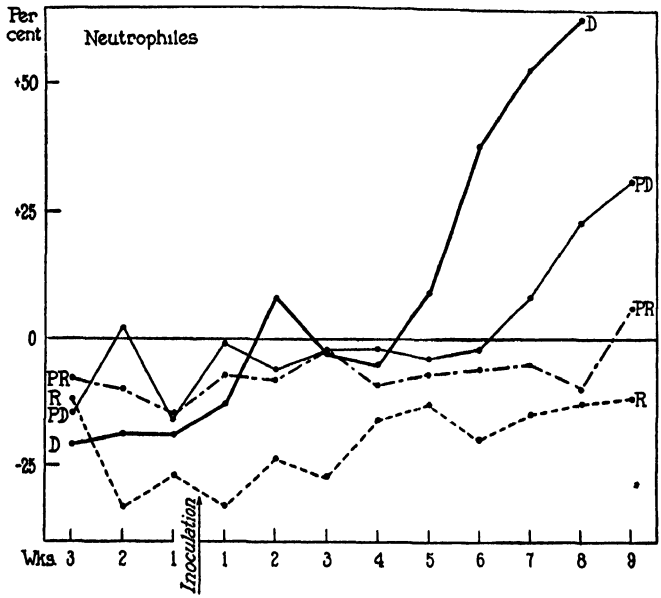
globin content, the excursions are more abrupt and the changes in level more pronounced. Before inoculation all four curves are above the base line which represents a value of 63 per cent; that of the probable death group contains one marked irregularity. After inoculation, three curves show an immediate drop followed by a rise. In the case of the deaths, a fairly constant level in the neighborhood of the base line is maintained for 5 weeks; during the next 3 weeks, the curve falls to 5, 7.5, and 2.5 per cent below the normal value. In the first half of the postinoculation period, the curve of the probable death group ranges between 3.5 and 7 per cent above the base line but during the last half, it falls steadily from this high level to a minus 5.5 per cent, a total change of 12.5 per cent. Despite irregularities, the curve of the recoveries shows a tendency toward the maintenance of higher values after inoculation. A similar tendency is seen in the probable recoveries until the last two observations when the direction of the curve appears to be definitely downward. It should be noted, however, that the values of both the probable recoveries and the recoveries are above the base line throughout the observation period while those of the deaths and probable deaths fall below the normal value during the latter part of this period.



TEXT-FIG. 3. Total white blood cells

White Blood Cells:—Before inoculation, the mean total white counts of all four groups of animals varied from 5 to 25 per cent below the

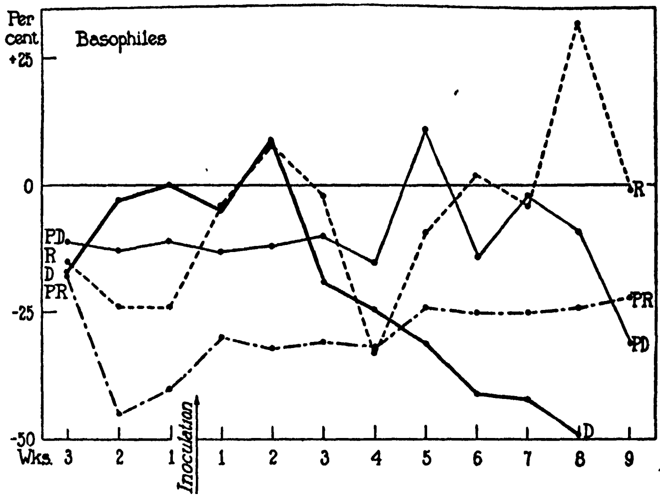
standard value of 9560 cells per c.mm.; the weekly variations of any one group, however, did not exceed 10 per cent of this value (Text-fig. 3). After inoculation, the trend in all groups was toward increased values throughout the period of observations as is shown by the upward direction of the curves. This trend was most marked in the case of the fatal group in which the highest levels were found during the last 3 weeks; the curve shows that a value of 25 per cent above the standard value was reached as compared with the average minus 15 per cent value of the preinoculation period. The curve representing the probable deaths shows a similar although slightly less marked change and in addition, it will be noted that the time of the rise occurred later. In the case of the probable recoveries, the findings were more irregular than with the deaths or the probable deaths, but as the general direction of the curve indicates, there was a similar tendency toward increased white counts which, however, were not as high as with either of the other groups. In the recovered animals, the total white cells showed a slight numerical increase after inoculation amounting approximately to 15 per cent above the preinoculation level. It will be noted, however, that at no observation did the total white count of the recovered group exceed the standard value while this was found to be the case with the other groups, particularly with the deaths and the probable deaths.



TEXT-FIG. 4. Neutrophiles (pseudo-eosinophiles)

Neutrophiles (Pseudo-Eosinophiles):—The preinoculation levels of the neutrophilic leucocytes in all four groups were below the standard value of 4340 cells per c.mm., the general group range being minus 10 to 25 per cent of this value (Text-fig. 4). The postinoculation observations showed that in the fatal group, the neutrophiles became increased, that this change became pronounced in the 5th week, and that a figure of 65 per cent above the standard value was reached at the time of the last examination. This value represents a rise of 85 per cent above the preinoculation level of the group. A similar but less marked increase to 30 per cent above the standard value occurred in the group of probable deaths and as will be seen by referring to the curves in Text-fig. 4, it occurred 2 weeks later than the decided rise of the neutrophiles of the fatal group. The curves representing the probable recoveries and the recoveries show that while there was also a tendency toward increased neutrophile counts after inoculation, the changes were comparatively slight. It is of interest to note, however, that the curve of the probable recoveries ends above the base line as was the case with the other two groups in which tumor was present

at the conclusion of the experiment. With the complete recoveries, on the other hand, the curve throughout lies below the base line.

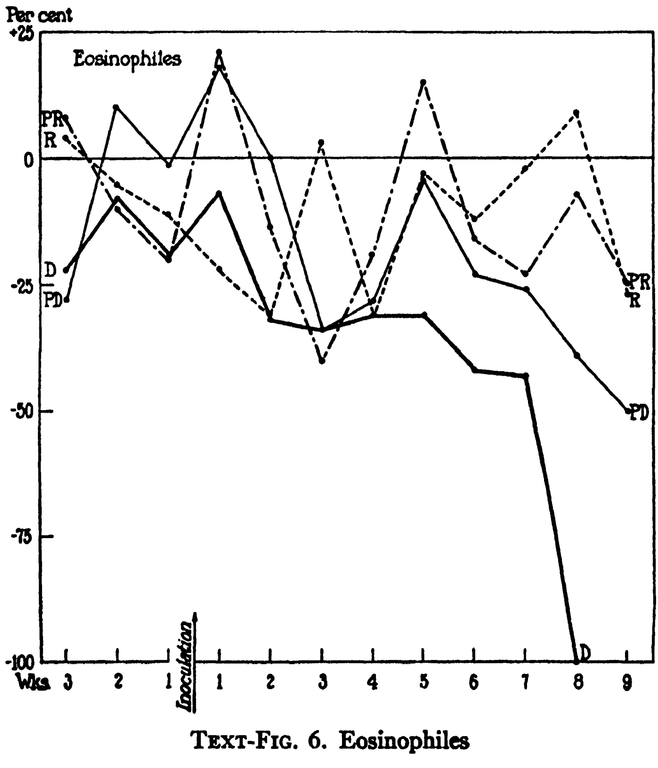


TEXT-FIG. 5. Basophiles

Basophiles:—The observations with respect to the basophiles are illustrated by the curves in Text-fig. 5. It will be seen by referring to these curves that there was considerable variation in the mean group levels of these cells during the 3 weeks prior to inoculation, the general range being from 5 to 25 per cent below the standard value of 950 cells per c.mm. In the groups of deaths and probable recoveries, moreover, the findings were comparatively irregular, the shifts in level amounting approximately to 15 and 25 per cent respectively. After inoculation it was found that in the case of the fatal group, the number of basophiles became markedly decreased, the change being pronounced at the 3rd week and continuing thereafter throughout the observation period; at the last examination, the count had fallen to 50 per cent below the standard value. With the probable deaths, a somewhat similar change was found after the 5th week of the disease, but it was less marked and less consistently followed as will be seen by comparing the respective curves. The last observation of the group of probable deaths was 30 per cent below the standard value as compared with the high point of plus 10 per cent at the 5th week and a

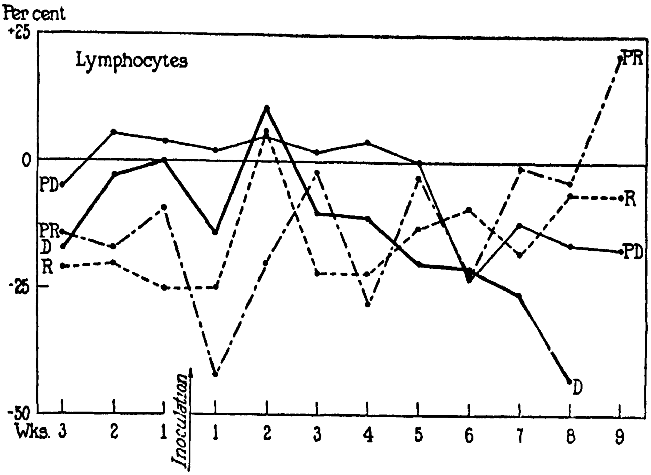
general preinoculation level of minus 10 per cent. In contrast with these findings, a sustained drop in the mean basophile count of the probable recoveries and recoveries was not observed. The curve representing the recovered group shows two major swings of considerable amplitude, but during the latter half of the observation period, that is, after the 4th week, the general trend is definitely upward and it ends on the base line. In the case of the probable recoveries, although the direction of the curve is slightly upward, it is maintained fairly constantly at levels of 30 and 25 per cent below the standard value.

It will be noted that during the latter half of the observation period, the basophile curves of the three groups of animals in which tumor was found at postmortem examination, all lie below the standard value while that of the recovered group in which no tumor was found lies at or above this value.



TEXT-FIG. 6. Eosinophiles

Eosinophiles:—The mean preinoculation eosinophile counts of all four groups were irregular. The limits of the irregularities as shown by the curves in Text-fig. 6 were 10.2 per cent above to 25.5 per cent below the standard value of 215 cells per c.mm.; the widest range of weekly variation of individual groups was 15 per cent of the standard value except in the case of the probable recoveries in which it was 35.5 per cent. After inoculation, the irregularities were generally more pronounced, a feature which is brought out by the pronounced swings of the curves. With the fatal group, there was a definite drop in the mean number of eosinophiles at the 2nd week; this new level was continued for three observations and was succeeded by even lower levels, and eventually at the last examination, no eosinophiles were found. The results in the other groups were not so clean-cut. In the probable deaths a similar but less pronounced decrease of eosinophiles occurred as shown by the general downward trend of the curve from its highest point in the 1st week after inoculation, although there is an upward swing during the 4th and 5th weeks corresponding to the stabilized period of low levels in the fatal group. With the probable recoveries and the recoveries, the results are not striking. It will be noted, however, that after the 4th week, the curves for these two groups are consistently higher than those of the deaths and the probable deaths, and that for the last four observations the mean numbers of eosinophiles in the recoveries were greater than in the probable recoveries. These features taken in conjunction with those relating to decreased values in the groups of deaths and probable deaths suggest that an increased eosinophile count is probably characteristic of the recovered state.



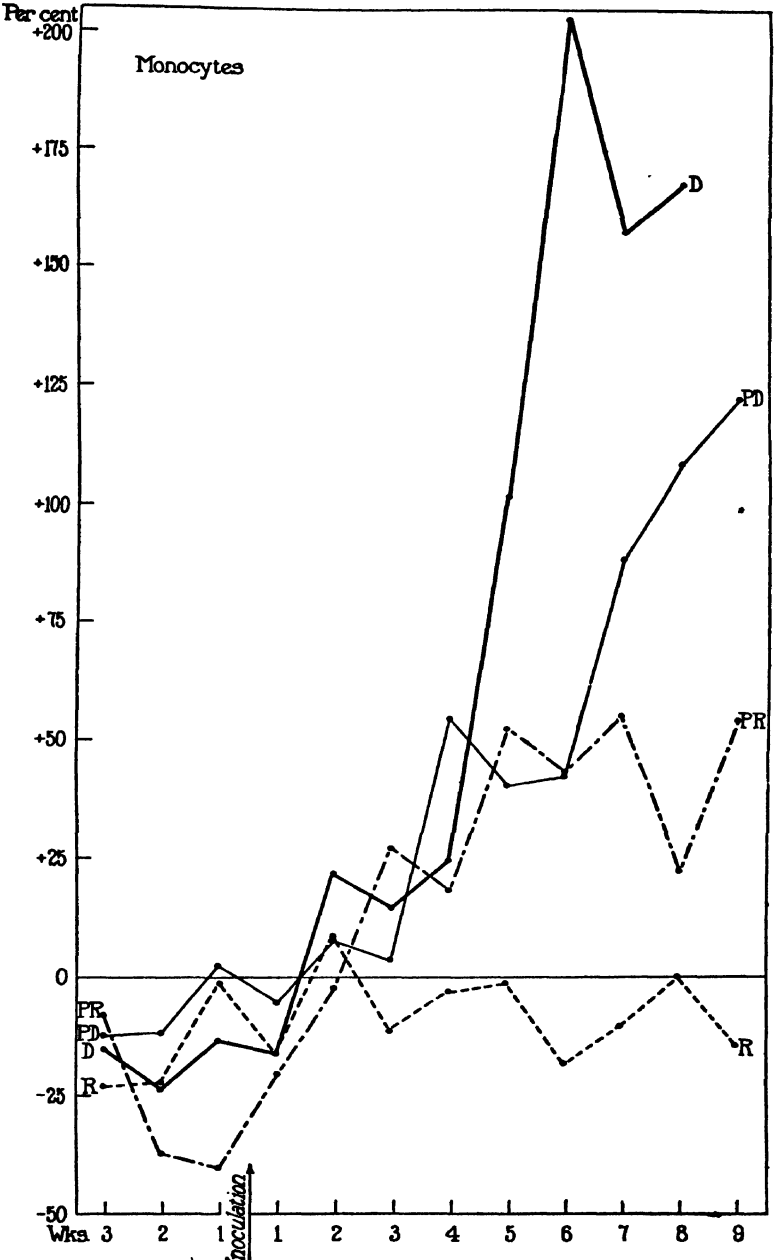
TEXT-FIG. 7. Lymphocytes

Lymphocytes:—The observations regarding the lymphocytes are illustrated by the curves in Text-fig. 7. It will be seen by referring to these curves that the average mean levels of the lymphocytes in the four groups of rabbits before inoculation were variable, their limits lying between the standard value of 3050 cells per c.mm. in the case of the probable deaths, to approximately 20 per cent below this figure in the case of the recovered group. Irregularities of individual groups ranged from 5 to 15.5 per cent on the standard value scale.

A week after inoculation, a drop in the mean numbers of lymphocytes was observed in two groups, the deaths and the probable recoveries, while in the others, little or no change was found; at the next observation, 2 weeks after inoculation, the numbers in three groups were considerably increased. From this time onward, the lymphocytes of the fatal group showed a steady numerical decline which is well brought out by the curve of Text-fig. 7; at the final observation, the level reached the low point of 40.5 per cent below the standard value as compared with an average preinoculation value of 5 per cent below and with 10 per cent above the standard value at the 2nd week after inoculation. In the case of the probable deaths, there was no significant change until the 6th week after inoculation. At this time, there

was a moderate decrease in the mean numbers of lymphocytes which persisted to the end of the observation period. The extent of the decrease amounted to approximately 20 per cent on the present scale as is shown by the curve in Text-fig. 7. The results in the probable recoveries and the recoveries are less definite. It will be seen, however, by referring to the curve representing the probable recoveries that despite frequent and comparatively wide variations, there was a distinct tendency in the latter half of the observation period for the lymphocytes to become increased over the average level of the preinoculation period and of the values found in the first weeks after inoculation. The curve rises from a point 25 per cent below the standard value at the 4th and 6th weeks after inoculation to a final level of plus 20 per cent. With the recovered group, the curve indicates that following the low levels of the 3rd and 4th weeks after inoculation, there was a fairly consistent trend in the direction of slightly increased lymphocyte counts which were still maintained at the last examination.

It would appear from these results that the mean number of lymphocytes in the peripheral blood tends to be somewhat decreased in rabbits succumbing to the effects of this neoplastic disease and that a similar but less marked effect is associated with a less severe process. On the other hand, the mean lymphocyte values tend to be slightly increased in rabbits in which the process is mild or in which recovery takes place.



TEXT-FIG. 8. Monocytes

Monocytes:—The observations on the monocytes are illustrated by the curves in Text-fig. 8. The preinoculation levels in all four animal groups were lower than the standard value of 1000 cells per c.mm. In the case of the deaths, the probable deaths, and the recoveries, successive weekly values showed variations of 10 to 20 per cent of the standard value; with the probable recoveries, the range extended to 30 per cent. The average levels in this period, as may be seen by the curves, were approximately 20, 7, 14, and 27 per cent below the standard value for the deaths, the probable deaths, the probable recoveries, and the recoveries respectively.

After inoculation, the monocytes in all groups except the recoveries, became markedly increased, that is, a peripheral blood monocytosis was associated with tumor growth. Beginning at the 2nd week, all four groups showed increased mean numbers of monocytes, the levels attained being higher than the standard value in three groups and equal to it in the fourth. In the case of the fatal group, this higher level was maintained for the following 2 weeks, and from this time to the end of the observation period, very large numbers of monocytes were found. 6 weeks after inoculation, the increase amounted to 200 per cent above the standard value; in the 7th and 8th weeks the figures were slightly smaller, but it will be remembered that these last two observations represent 10 and 3 animals (the most resistant of the group) as compared with 17 at the 6th week. The findings with respect to the group of probable deaths were comparable to those of the fatal group as is shown by the respective curves. It will be noted, however, that the time of the first marked monocytic increase occurred later, that is, at the 7th as compared with the 5th week in the fatal group, and furthermore, that the magnitude eventually attained at the end of the observation period was not as great, that is, 125 as compared with 170 per cent. The findings of the last three observations in the probable death group show progressively larger numbers of monocytes as is illustrated by the regularly rising curve, and this feature is undoubtedly influenced by the fact that each observation represents the same number of animals, none dying before the end of the experiment as was the case with the fatal group.

In the probable recoveries, increased numbers of monocytes were observed to the extent of 50 per cent above the standard value. This

condition was attained by the 5th week after inoculation and corresponds to a similar level on the part of the probable death group and to a value of plus 100 per cent for the fatal group. During the last four observations, the curve representing the probable recoveries is irregular but on the whole, it tends to remain at the level of 50 per cent above the standard value. In the case of the recovered animals, the highest monocyte level observed was at the 2nd week after inoculation, that is, 9 per cent above the standard value. At subsequent examinations to the end of the observation period, there was an irregular decrease in the mean numbers of monocytes, and it will be noted that after the 2nd week, the curve does not again rise above the base line and that its general contour resembles its preinoculation portion.

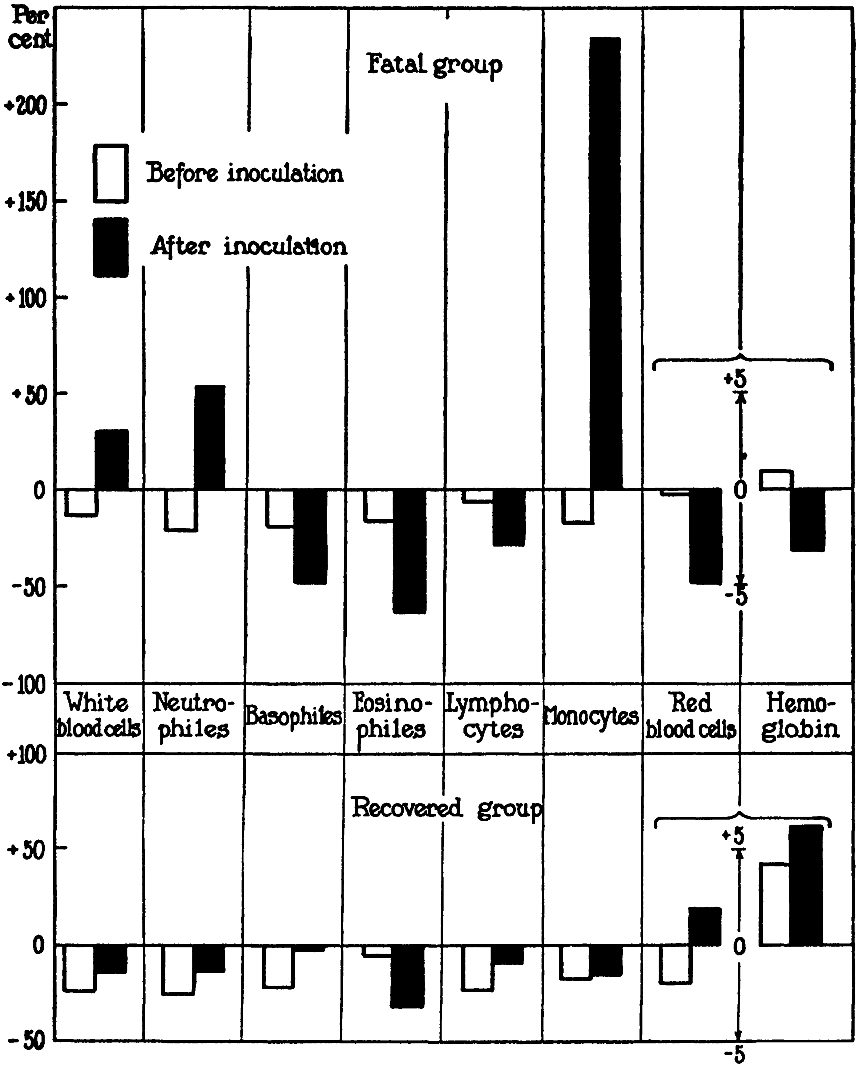
The above discussion of these results may be summarized as follows: During the course of this particular neoplastic disease, certain alterations of the peripheral blood picture were observed. The most striking changes during the course of a fatal or probably fatal condition concern the monocytes, a pronounced numerical increase of which was found, and secondly, the eosinophiles, the numbers of which were markedly reduced. In the case of the basophiles, a change similar to but less marked than that of the eosinophiles was observed (12). The relation of the numbers of lymphocytes to the course and type of disease was less definite than that of the monocytes, eosinophiles, and basophiles, but the findings showed a general correspondence to those of the eosinophiles and basophiles rather than to the monocytes. In the case of the neutrophiles, it was found that their numbers became increased in an actively progressing disease. As would be expected, the character of the total white count was greatly influenced by the neutrophiles. The very large numbers of monocytes in the fatal and the probable death groups also contributed to the increased total white counts. And finally, in conditions of pronounced or well marked malignancy, decreased numbers of red cells and a lowered hemoglobin content were observed; the change in the red cells preceded that of the hemoglobin.[4]

[4] In connection with these results in cases of well marked malignancy, it is of interest to recall that the blood count of the original tumor rabbit taken shortly before death showed the characteristic findings here described (10).

The time of these changes should be referred to. In the fatal group, the curves representing the red blood cells, the hemoglobin content, the various classes of white cells, and the total white count show a decided trend of direction at the 4th and 5th weeks after inoculation although the trend itself may have been initiated earlier, as was the case with the monocytes. It will be noted in the group of probable deaths that, on the whole, the changes which were of the same general character as those of the fatal group, occurred somewhat later. This finding is in keeping with the differences in malignancy level of the two groups. In this connection, it should be mentioned that although the rate of growth of the primary tumor and the distribution and growth of metastatic tumors vary widely, postmortem examination of a large number of rabbits at variable periods of the disease has shown that by the 3rd or 4th week after inoculation, the tumor process is well established in cases of pronounced or average malignancy.

As far as the probable recoveries and recoveries are concerned, the results in general correspond to what might be expected in the light of the findings in the groups characterized by an actively progressing tumor process. Thus, in the case of the monocytes which is the most striking example, increased counts of a moderate degree were observed in the group of probable recoveries, but there was little change in the group of recoveries.

The differences in the blood picture of rabbits representing the two extremes of reaction to the malignant disease, that is, the fatal and the recovered cases, are brought out by the accompanying graphs in which the last observations of these groups are illustrated (Text-fig. 9). For the recovered group, the values are, of course, those given in the curves of the text-figures; for the fatal group, on the other hand, the values are different since they represent the combined last observations of each animal irrespective of the time of death.



TEXT-FIG. 9. Last observations on the fatal and recovered groups. Values are expressed in the form of mean percentage deviations (algebraic sum) from standard values.

The outstanding changes which have been noted in the present experiments far exceed the spontaneous variations found in groups of normal rabbits examined for comparable periods of time (2, 3, 4, 5). Strictly speaking, the present results cannot be compared with those of the normal animals because they are derived from an analysis in which the findings of individual rabbits from different groups are combined, while with the normal animals the entity of each group was preserved. It may be stated, however, that an analysis of each animal group of these experiments has been made and that the results are substantially the same as those presented in this paper.

It should be mentioned that all the characteristic blood picture changes associated with the malignant process as determined by the analysis of the material on a combined group basis, were not invariably found in individual animals; in certain cases, one or more of these changes were present while others were lacking. These instances were rare, however, when one takes into consideration the general character of the blood counts over the entire observation period. There was one striking example of a fatal case with a widespread distribution of tumor in which the monocytes and neutrophiles were not increased, the lymphocytes were unchanged, and the basophiles were only slightly diminished; there was, however, a decreased red count, a lowered hemoglobin content, and a decreased eosinophile count.

At the present time, no interpretation of the results of these experiments has been made. It is evident that in rabbits in which active and extensive tumor growth takes place, that is, in animals with a low resistance to the disease, the numbers of peripheral basophiles, eosinophiles, and probably the lymphocytes are low in contradistinction to the high numbers of monocytes and neutrophiles. But whether these states are the result of the tumor process or are more intimately connected with the cause of its activity, cannot now be determined. The results of the tissue studies carried out in connection with observations on the peripheral blood as well as those bearing on the character of the blood picture before inoculation may throw some light on the subject.

SUMMARY AND CONCLUSIONS

Successive blood counts at weekly intervals were made on rabbits inoculated with a transplantable malignant neoplasm of epithelial origin. There were 78 animals distributed in 9 groups; the period of observation after inoculation was 2 months. The results have been considered with respect to the character of the tumor process as determined by postmortem examination, the animals being classified as deaths, probable deaths, probable recoveries, and recoveries. The blood findings have been analyzed on the basis of the values derived from normal rabbits. A comparison has also been made with the results of preinoculation counts.

In rabbits in which the tumor process was of pronounced or well marked severity, the numbers of monocytes became greatly increased; the neutrophiles and the total white blood cells were also increased; the eosinophiles, and to a less extent, the basophiles and lymphocytes were decreased; the red cell count was decreased and the hemoglobin content was lowered.

In rabbits which recovered from the tumor inoculation, there were no outstanding changes in the blood picture when the findings for the entire observation period were considered. In the group of probable recoveries, the results were variable; there was, however, a definite although moderate increase of monocytes, and in general, the findings reflected the mildness of the disease when compared with those of the fatal cases and the group of probable deaths.

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4. Pearce, L., and Casey, A. E., *J. Exp. Med.*, 1930, **52**, 145.
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10. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, **37**, 61 ff.
11. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, **37**, 799.
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EXPERIMENTAL POLIOMYELITIS

HISTOLOGY OF THE PERSISTENT LESIONS OF THE CENTRAL NERVOUS SYSTEM

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The histopathology of acute poliomyelitis in both man and monkey has been repeatedly and thoroughly discussed, whereas the stage of recovery and repair has received little attention in the literature. Although residual lesions in the central nervous system of human cases of long standing have been described, the necropsy material has been scarce, since the death of patients during that period depended not on the poliomyelitic infection but on intercurrent illness and accident. Many years of investigations on the monkey have shown a close correlation between the clinical manifestations of the experimental disease in these animals and with those in man, but owing to the severity of the infection, death tended to take place in the acute stages or before recovery had proceeded very far.

It was possible, in this laboratory, to keep fifteen *Macacus rhesus* monkeys alive for periods varying from 19 to 309 days after the onset of symptoms. Careful nursing during the prostrate paralytic period was often necessary to maintain nutrition and to avoid decubitus, and while some animals died despite these measures, others regained full functional activity of all four extremities. A study was therefore made of all the variations of disease and recovery of the central nervous system in order to determine the persistence of active inflammatory lesions and the rate of progressive repair, in the thought that such information might be of value in its application to human poliomyelitis from the point of view of prognosis and treatment.

REVIEW OF THE LITERATURE

The typical pathologic changes in cases of acute poliomyelitis have been described by many authors, notably by Medin,[1] Wickman,[2] Zappert, von Wiesner and Leiner,[3] Römer,[4] Flexner and Lewis,[5] Landsteiner[6] and more recently by Hurst.[7] Essentially, these investigators agree regarding the histopathology of the disease, although there have been differences in the interpretation of the origin and significance of the lesions themselves.

The chronic stages, on the other hand, have received little attention. Schwalbe[8] summarized the clinical and pathologic reports of fourteen cases of chronic poliomyelitis, collected from the literature prior to 1902, which came to autopsy at periods varying from five months to sixty-five years after the onset of the paralysis. From his outline it appears that scattered lesions in the cords were present that had not given rise to clinical signs, that cicatrization was noted as early as the fifth month, that perivascular lesions were found in two cases of five months' and two years' duration respectively, and that obliteration and degeneration of the nerve cells were universally present in focal areas.

Schwalbe also reported a case of approximately four months' standing which he considered characteristic of the "reparative" phase. The patient had suffered from acute poliomyelitis before death occurred from an intercurrent disease. Clinically, only the left leg gave evidence of involvement, but the damaged area penetrated higher and lower levels than had been suspected, while a focus of degeneration which had given no symptoms was found in the right lumbar cord. Grossly there was no shrinkage of the anterior horns characteristic

1. Medin, O.: Hygiea 52: 657, 1890.
2. Wickman, I.: Die akute Poliomyelitis beziehungsweise Heine-Medinsche Krankheit, Berlin, Julius Springer, 1911.
3. Zappert, V.; von Wiesner, R., and Leiner, K.: Studien über die Heine-Medinsche Krankheit, Vienna, Franz Deuticke, 1911.
4. Römer, P.: Die epidemische Kinderlaehmung, Berlin, Julius Springer, 1911.
5. Flexner, S., and Lewis, P. A.: J. Exper. Med. 12: 227, 1910.
6. Landsteiner, K., in Kolle, W.; Kraus, R., and Uhlenhuth, P.: Handbuch der pathogenen Mikroorganismen, Jena, Gustav Fischer, 1929, vol. 8, p. 38.
7. Hurst, E. W.: J. Path. & Bact. 32: 457, 1929.
8. Schwalbe, E.: Beitr. z. path. Anat. u. z. allg. Path. 32: 485, 1902.

of scar formation, but on section the gray matter was found to retract from the cut surface. On microscopic examination, the areas of destruction were seen to be localized in the central portion of the anterior horn and showed a tendency to spread upward and inward rather than laterally. The nerve cells about the outer border showed the better state of preservation where the entire anterior horn was not involved. Schwalbe remarked on the patchiness and inconsistency of the lesions at adjacent levels in the cord, and described changes in the nerve cells of four types: (1) pale cells with visible nuclei, faint processes and loss of Nissl substance; (2) shrunken, darkly staining cells, which occasionally contained granular cytoplasm, but in which the nuclei and processes were no longer discernible; (3) cells in all of the stages between those described and normal cells (these were usually seen at the borders of the lesion) and (4) "calcified" cells. There was found to be no direct relation between destruction of the nerve cells and the perivascular reaction since vascular adventitial spaces filled with fat and fat containing cells were seen in one posterior horn and in the anterior horns above and below the levels at which other abnormalities were demonstrable. Hyperemia and proliferation of vessels were noted. The perivascular infiltrate was described as consisting of cells containing fat and round cells. The latter were also observed in the gray matter about the areas of destruction, together with clumped "Fettkoernchenzellen." Fat was seen lying in the "spaces" around the nerve cells and about the nerve fibers in the anterior roots, anterior columns and the gray commissure.

No disturbances of the fiber tracts were noted, but there was a loss of neurofibrils in the anterior roots and in the areas of destruction in the gray matter, in which Schwalbe noted a fine glial network which suggested a condition similar to that of "gliosis." As there was no increase in glia nuclei, and as fat-containing cells were often seen, he concluded that in all probability there was no true glial proliferation and that the scar formation of the late "residual" stage had not set in.

A number of cases of chronic human poliomyelitis were reported between 1902 and 1911, but after the successful inoculation of monkeys with the virus, in 1909, by Landsteiner and Popper,[9] far more atten-

9. Landsteiner, K., and Popper, E.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 2: 377, 1909.

tion was given to the acute phases of the disease. In only a few laboratories were pathologic studies undertaken on the recuperative changes.

Levaditi and Stanesco (1910)[10] discussed the lesions in three monkeys that had survived the onset of the disease for periods of twenty-one, twenty-nine and sixty-seven days. Two of these suffered from a paraplegia, one from monoplegia. Only the third showed mononuclear and polymorphonuclear cells in the cortical meninges together with cerebral perivascular infiltration. In the brain stem, active inflammatory areas were found. In the cervical and thoracic regions of the cord no pathologic signs were present, but the lumbar segments showed signs of a less active process, in which practically no nerve cells remained in the anterior horns, perivascular lesions persisted, and the tissues were infiltrated with mononuclear cells. It was thought that these lesions corresponded to the clinical observations in that only unilateral damage was present in the anterior horn in the case with monoplegia, while peripheral nerve degeneration corresponded with the affected spinal levels as did the atrophic muscles supplied by them. In contradistinction to acute poliomyelitis, no lesions of the meninges or vessels of the white matter were seen in the cords, while in the gray matter there were no polymorphonuclear cells, and "chronic inflammation" was characterized by mononuclear infiltrates about the vessels and in the tissues. The posterior horns were spared.

The presence of acute lesions in the brain stem was regarded as a sign of a recurrence in that location after the process of the disease in the lumbar area had passed the phase of active destruction. This condition was thought to be due to the slow development of an immunity to the infection. The predilection of the virus for the lumbar cord, regardless of the route of inoculation, was also pointed out.

In 1911, Römer[4] described the stage of repair in a *Macacus rhesus* monkey that showed a paraplegia persisting until death thirty-six days after the onset of the disease. The brain was not examined. Although no lesions were found in the cervical and dorsal spinal cord, bilateral involvement of the anterior horns of the lumbar region was

10. Levaditi, C., and Stanesco, V.: Compt. rend. Soc. de biol. **68:** 664, 1910.

observed, characterized by widespread destruction without signs of the formation of scar tissue or vascular proliferation. The fibrous structure was described as "porous," so that only a fine fibrillar network, studded with small clumps of round cells, interlaced the meshes. Perivascular infiltration was present, and nerve cells were almost completely absent. Glial proliferation and Fettkoernchenzellen were remarked as characteristic of the process of repair. Four other animals were also reported, which recovered completely without traces of residual paralysis, and in which histologic examination revealed no lesions whatever in the nervous system. A fifth monkey had made a distinctly progressive recovery following paraplegia and paralysis of the upper extremities, only to relapse on the twentieth day after the onset of the symptoms, so that quadriplegia was followed by death two days later. In this case the spinal cord showed typical lesions of acute poliomyelitis.

Römer's description of the residual stage of the human disease agreed with that of Müller (1911)[11] who stated that a thick scar was ultimately produced in the damaged areas by glial proliferation after the elimination of detritus by "Koernchenzellen." A cross-section of the cord showed the anterior horns to be grossly shrunken and depressed. Microscopically, the neighboring tracts and anterior roots were found to be atrophic. Degeneration of the other fiber tracts was also mentioned together with secondary changes in the central convolutions of the brain in longstanding cases in which atrophy of the extremities had persisted.

In the more recent literature, there have been remarkably few references to the pathology of chronic poliomyelitis. Hurst[7] reported one case in a *M. rhesus* monkey that was intracerebrally infected with the "Ay" virus, and in which acute symptoms developed: double ptosis, paralysis of the right arm and paresis of both legs. The animal showed partial recovery of function in the affected limbs, and was killed on the thirty-fifth day after the onset. On pathologic examination, no gross abnormalities were noted in the central nervous system. Microscopically, a marked loss of neurons was evident at the affected levels, but the remaining nerve cells were normal in appearance.

11. Müller, E.: Handb. d. inn. Med. 1: 840, 1911.

There was no perivascular or meningeal reaction. In the involved areas, there was a definite increase in neuroglia cells which were large and had many processes, but no definite fibril formation was observed. The microglia also showed proliferation, representing all of the forms from normal cells to compound granular corpuscles laden with fat. The latter were seen in the greatest number lying about the capillaries and small vessels, or, in the case of the granular corpuscles, in the perivascular spaces. Lipoid staining material was also found in the tissues of the anterior horns. The myelin sheaths were reduced in number, ballooned and irregular in their staining reaction, while degeneration was seen in the medullated fibers leading to the anterior roots, where no degeneration of the fiber tracts was observed. The median nerves showed advanced myelin destruction. Other regions of the central nervous system proved to be entirely normal.

EXPERIMENTAL WORK

Fifteen *Macacus rhesus* monkeys, previously used for various experiments on poliomyelitis in this laboratory, had been carefully nursed through the acute phases of the disease to partial or complete stages of functional recovery. They had been infected by intracerebral inoculation, by nasal instillations or by injections of virulent material into the lumbar or cisternal theca. These procedures have been described elsewhere[12] and will not be discussed here. The M.A. virus, referred to by Hurst[7] as "A.M.," was a weak strain, as were the Ay and Mt. S. viruses. The first of these was obtained from the lumbar cord in a human case of poliomyelitis, in 1909, which at that time was passed to monkeys by Flexner and Lewis;[13] the second was a human strain passed to monkeys by Aycock and Kagan,[14] whereas, the third was a pooled virus from parts of the central nervous system of three patients with acute poliomyelitis, sent to The Rockefeller Institute from the Mount Sinai Hospital in New York. In general, the animals showed a tendency toward more complete functional rehabilitation after infection with these three viruses than after inoculation with the strong PMV[15] strain, designated as

12. Rhoads, C. P.: J. Exper. Med. 53: 115, 123 and 137 (Jan.) 1931; 53: 399 (March) 1931.

13. Flexner, S., and Lewis, P. A.: The Transmission of Acute Poliomyelitis to Monkeys, J. A. M. A. 53: 1639 (Nov. 13) 1909.

14. Aycock, W. L., and Kagan, J. R.: J. Immunol. 14: 85, 1927.

15. PMV is a pooled monkey virus consisting of a mixture of the M.A. and K strains. The latter was derived from a human spinal cord in 1909. Flexner, S., and Lewis, P. A.: The Transmission of Poliomyelitis to Monkeys, J. A. M. A. 53: 1913 (Dec. 4) 1909.

"Fl" by Hurst, which was followed by subacute or chronic poliomyelitis, although it is probable that the animals in cases 3, 4 and 8 might have rallied further had the period of convalescence been prolonged.

The clinical data are incomplete from the neurologic point of view, owing to the fact that the present study was undertaken after the completion of the experiments in which the animals were originally used, and after the majority of them had been killed, so that it was impossible to correlate the pathology of the central nervous system with the symptomatology. In the protocols in table 1 the term "prostrate," unless otherwise modified, signifies a condition in which there was partial or complete quadriplegia together with paralysis of the bladder. The animals were generally able to move their heads, and mastication and deglutition showed no obvious disturbances. Facial paralyses were usually early and transitory. The condition referred to as "complete functional recovery despite residual atrophy" was one in which the monkeys climbed about freely, although there was limitation of motion of the hind legs, and, to a lesser extent, of the upper extremities, owing to contractures and atrophy of various groups of muscles.

It will be seen from these protocols that case 1 survived for nineteen days but ran a progressively downhill course comparable with that of the severe acute cases of shorter duration. It has been included in this series as illustrative of the transition between the acute condition described by Hurst and the later group dealt with here.

Method

Autopsy was performed on the animals as soon as possible after death, or immediately following etherization and exsanguination. The central nervous systems were fixed and stained as follows:

Formaldehyde Fixation (10 per cent).—Frozen sections were stained for fat by Fettpenceau and hematoxylin, and Fettpenceau and silver impregnation; for macroglia, by Cajal's gold sublimate impregnation; for microglia and oligodendroglia, by Penfield's combined method,[16] and for neurofibrils, by Bielschowsky's method and Cajal's silver impregnation.[17]

In the last named method, the sections were cut at 25 microns, received in distilled water and washed well. Five or six sections were then placed in 10 cc. of a 2 per cent silver nitrate solution to which 6 drops of pyridine had been added, and warmed over an alcohol flame for from thirty minutes to three hours, depending on the completeness of impregnation at various intervals of time. The amber brown sections were then dipped in 95 per cent alcohol and transferred to Cajal's

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16. Penfield, W., and Cone, W.: Neuroglia and Microglia (The Metallic Methods), in McClung, C. E.: Handbook of Microscopic Technique, New York, Paul B. Hoeber, 1929.

17. Ramon Cajal, S.: Trav. du lab. de recherches biol. de l'Univ. de Madrid 22: 157, 1924.

TABLE 1

Protocols of Monkeys with Poliomyelitis

No.	Inoculation			Virus	Clinical Course	Days
	Date	Route	Dose, Cc.			
1	4/13/29	Cistern	0.005	PMV 5% F.F.*	4/28/29, tremor, ataxia; 4/29/29, paralysis of shoulder girdles and vocal staccato, from 4/30/29 prostrate, to 5/15/29, slowly progressive weakness; 5/16/29, found dead	19
2	12/ 3/28	Cerebral	1.0	Mt. S. 10% F.B.†	From 12/14/28 to 12/26/28, tremor, slight ataxia and excitement; 12/26/28, paralysis of the right shoulder girdle; 12/27/28, paralysis of the left shoulder girdle; 12/28/28, paresis of both legs and generalized weakness; 12/29/28, paralysis of the right arm and progressive weakness; 12/31/28, more active, with general condition improved; 1/2/29, further improvement and some functional recovery of the muscles; 1/11/29, etherized	29
3	4/15/29	Cistern	0.01	PMV 5% F.F.	4/19/29, ataxic, slow; 4/20/29, asymptomatic; 4/25/29, generalized convulsion; 4/27/29, paralysis of the right arm and tremor; 4/28/29, ataxia, paralysis of the left arm and paresis of both legs; 4/29/29, prostrate; from 4/30/29 to 5/28/29, prostrate, bright, slight movement of the left arm and head; able to raise head and shoulders and to pull itself about in the cage; etherized	40
4	10/ 1/29	Cerebral	0.02	PMV 5% F.F.	10/20/29, right facial paralysis and bilateral paresis of the shoulder girdle, excitement, tremor, ataxia; 10/11/29, paralysis of both shoulder girdles, progressive weakness; 10/12/29, paresis of the legs, able to sit up; 10/13/29, paralysis of the legs; unable to sit up, but crawls about; from 10/16/29 to 11/19/29, brighter; able to move head and shoulders; finally able to move arms and pull itself about in the cage; considerable improvement; etherized	41

5	From 10/17/29 to 10/19/29	Nasal	3 in 3 days	PMV 10% G.‡	10/26/29, paralysis of the right leg and paresis of the shoulder girdles; tremor and ataxia; from 10/27/29 to 12/11/29, prostrate but bright; slight recovery of function; marked atrophy and contractures; etherized	47
6	From 10/17/29 to 10/19/29	Nasal	3 in 3 days	PMV 10% G.	10/26/29, excitement; 10/27/29, paralysis of both legs, staccato voice, tremor and ataxia; from 10/28/29 to 12/11/29, prostrate but bright; quadriplegia except for paresis of right shoulder girdle; recovery very slight; marked atrophy and contractures of all the extremities; etherized	47
7	3/ 5/29	Cerebral	0.5	PMV 5% F.B.	3/11/29, paresis of both shoulder girdles, excitement, tremor and ataxia; from 3/12/29 to 4/26/29, prostrate but bright; gradual recovery; able to move head, shoulders and trunk; refused food; 4/27/29, found dead; postmortem examination showed severe anemia	48
8	From 9/20/29 to 9/23/29	Nasal	3 in 3 days	PMV 10% G.	9/29/29, excitement, tremor and ataxia; 9/30/29, paralysis of both shoulder girdles and double ptosis; 10/1/29, paresis of the left arm and both legs; 10/2/29, prostrate but bright; from 10/4/29 to 11/19/29, slight improvement; partial functional recovery of all four extremities; etherized	52
9	5/31/28	Cerebral	0.2	Ay. 5% F.B.	6/6/28, left facial paralysis; slight ptosis, excitement and head tremor; 6/7/28, paralysis of shoulder girdles and back, ataxia; 6/8/28, paralysis of both legs; 6/9/28, prostrate but bright; 7/21/28, very slight recovery; able to lift head; all limbs atrophic and spastic; edema and decubitus; 8/3/28, dying; etherized	50
10	2/ 5/29	Cerebral	0.01	PMV 5% F.F.	2/15/29, paralysis of the left shoulder girdle, excitement, tremor and ataxia; 2/16/29, paralysis of both arms and shoulder girdles, paresis of the left leg and back; 2/17/29, prostrate but bright; from 2/20/29 to 4/26/29, able to raise head but generally weaker; slow improvement, able to move head, neck and arms and to pull itself about in cage; poor nutrition; 4/27/29, found dead; postmortem examination revealed severe anemia	72

TABLE 1—*Concluded*

No.	Inoculation			Virus	Clinical Course	Days
	Date	Route	Dose, Cc.			
11	10/ 3/28	Cerebral Peritoneal	1.00 15.00	Mt. S. 10% F.B.	10/12/28, paresis of the left shoulder girdle and both legs, diarrhea; 10/13/28, paresis of the right shoulder girdle and back; 10/14/28, paralysis of the left shoulder girdle and legs, diarrhea; 10/15/28, paralysis of the left arm, right shoulder girdle and both legs, tremor; from 10/16/28 to 10/30/28, slightly more active; almost complete recovery of function despite residual atrophy of all four extremities; 1/3/29, emaciated; severe diarrhea; etherized	84
12	5/16/28	Cerebral	0.2	Ay. 5% F.F.	5/27/28, excitement, tremor and ataxia; from 5/28/28 to 5/31/28, paralysis of both arms and paresis of the back; prostrate but bright; from 6/1/28 to 1/23/29, prostrate and weak; complete functional recovery despite residual atrophy; etherized	242
13	5/ 3/28	Cerebral	0.1	Ay. 5% F.F.	5/10/28, left facial paralysis, vocal staccato and ataxia; 5/11/28, paralysis of shoulder girdles, paresis of the legs and back, tremor; 5/12/28, paralysis of the left arm and the right leg; from 5/13/28 to 1/23/29, paralysis of left leg; complete functional recovery despite residual atrophy; etherized	259
14	4/23/28	Cerebral	0.3	Ay. 5% F.F.	5/2/28, excitement; 5/3/28, paresis of the left leg, tremor and ataxia; 5/4/28, paresis of lumbar spine; from 5/5/28 to 5/31/28, paresis of the right leg and the right shoulder girdle; almost complete functional recovery despite residual atrophy; 1/23/29, etherized	267
15	3/ 3/28	Cerebral	0.3	M.A. 5% F.B.	3/21/28, paresis of the right shoulder girdle, excitement and ataxia; 3/22/28, paralysis of the right shoulder girdle and paresis of the left leg, tremor; 3/23/28, paresis of lumbar back; 3/24/28, paresis of the right arm; 1/23/29, complete functional recovery despite residual atrophy; etherized	309

* F.F. = fresh brain filtrate. † F.B. = fresh brain suspension. ‡ G. = glycerolated brain suspension.

reducer for two minutes (hydroquinone, 0.2 cc.; formaldehyde solution [Merck], 30 cc., and distilled water, 80 cc.). After thoroughly washing the sections in distilled water, they were fixed in sodium hyposulphite, dehydrated and mounted in Canada balsam.

This method was found to be simpler than the Bielschowsky impregnation, but consistently good results were not obtained by either technic. There were such marked variations, even in the normal control animals, that when the sections showed questionable pathologic changes with the routine stains, these were disregarded in the presence of an intact fibrillar network, while the converse situation was considered significant only when the unaffected cells gave evidence of complete impregnation. Sufficiently good preparations were made in some cases to permit a study of the various phases of neurofibrillar degeneration.

Paraffin sections were stained for cellular structure with hematoxylin and eosin; celloidin sections for cellular structure with hematoxylin and eosin, and for nerve fibers and myelin by the Kulschitsky-Weigert method.

Zenker Fixation.—Paraffin sections were stained for cellular structure with eosin-methylene blue by Mallory's method, substituting phloxine for eosin, and for fibrous structure with phosphotungstic acid and with aniline blue by Mallory's methods.

Alcohol Fixation (95 per cent).—Celloidin sections were stained for cellular structure with cresylecht violet (the results obtained with this stain were not entirely satisfactory).

Formaldehyde-Ammonium-Bromide Fixation (Cajal).—Frozen sections were stained for microglia and oligodendroglia by an unpublished modification of Hortega's silver carbonate method by Hortega.[18] This method was found to give consistently good results and to impregnate the oligodendroglia cells more satisfactorily than Penfield's combined method. Counterstains for fat (Fettpenceau) were made which clearly showed its intracellular and extracellular distribution. The nerve cells were in many cases weakly impregnated with silver, which facilitated the study of their relationship to the glia cells.

The blocks were removed from the fixative between the sixth and the eighth day and were heated to from 45 to 50°C. in formaldehyde-ammonium-bromide for from five to ten minutes. After cooling and rinsing in distilled water, frozen sections were cut at 15 microns. These were received in distilled water and immediately transferred to strong ammonia where they were allowed to remain, in a dark bottle, for at least one hour. One or two sections were washed rapidly in distilled water, placed in a small covered dish of Penfield's strong silver solution[16] for from two to five minutes, and transferred to 1 per cent formaldehyde solution where they became a deep, uniform brown. After washing in distilled water, the sections were toned in cold yellow gold chloride (1:500), fixed in 5 per cent sodium hyposulphite, dehydrated and mounted in Canada balsam.

Macroglia were stained according to Cajal's gold sublimate method, after fixation in formaldehyde-ammonium-bromide.

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18. Personal communication from Dr. Lawrence Kubie.

Penfield's modification[16] was not used, as it was found that satisfactory results could be obtained with sections treated in the same way as in the modified silver carbonate method already described. After cutting the sections, those to be impregnated with gold were placed in weak ammonia over night, after which the same technic was used as with the formaldehyde-fixed material. In this way the astrocytes, microglia and oligodendroglia could be studied in sections cut from the same block.

In some cases the whole nervous system had not been fixed, while in many others all the material had been fixed in formaldehyde. Only two monkeys were killed after the present study was undertaken, and on these all the staining methods described were used throughout the entire nervous system. In other instances the technic was limited by the original fixation. Four cases were available for complete investigation on blocks fixed in formaldehyde-ammonium-bromide, although gold sublimate and Penfield's combined impregnations[16] were made on several spinal cords which had been hardened in formaldehyde.

Histologic Observations

The histopathology of acute poliomyelitis in *Macacus rhesus* monkeys has been recently restudied by Hurst[7] who grouped his observations under the headings of pial infiltration, perivascular and extra-adventitial infiltration, diffuse and focal tissue infiltration, and nerve cell destruction. These lesions were discussed in respect to their distribution in the spinal cord, medulla and pons, midbrain, basal ganglia, cortex, cerebellum and other regions. This classification has been followed as closely as possible in order to facilitate comparison between the acute and chronic or recovered cases. The examination of the spinal cord has been subdivided into the lumbar, thoracic and cervical regions, and an attempt has been made to deal somewhat more specifically with the cortical lesions. No detailed description of the nuclei of the cranial nerves has been undertaken, since the clinical data were too incomplete to warrant an attempt at correlation with the pathologic observations.

It was possible to study the spinal cords of fifteen monkeys, the medullas of fourteen, the midbrains of eight, the basal ganglia of fifteen, the cortices of nine, the cerebella of nine and the intervertebral ganglia of nine, and to compare them with normal controls and with material from monkeys killed during the acute stages of poliomyelitis. In some instances only single sections through each area were available, and as the lesions are notably patchy in distribution, this report on the pathologic observations is in certain respects incomplete. Nevertheless, the material at hand presented a consistent picture, and suggested certain conclusions.

Spinal Cord

No matter what the route of infection, it was found that with the exception of one case, the lumbar cord was most severely damaged and showed the greatest degree of active inflammation. The thoracic region was not spared to any appreciable extent, and examination of several segments showed a patchy, although fairly consistent, transition between the conditions present in the lumbar and cervical cords. The latter tended to show an arrested or reparative picture in the cases of long standing.

Pial Infiltration.—While Hurst[7] found a marked generalized meningeal reaction scattered over various levels and surfaces of the spinal cord at the height of the disease process, he made the statement that "until a fairly late stage pial infiltration is insignificant and confined to a light excess of cells in the deeper part of the opening of the anterior fissure, or around the vessels of this region, to the entry zone of the posterior nerve roots, or to the meninges over the posterior septum." It is interesting to note that this distribution of lesions at the onset corresponds with that of the later period in those instances in which any meningeal reaction was discernible. Although the monkey in case 1 survived for nineteen days, the histologic picture here and elsewhere was entirely comparable with those in the severe acute cases of shorter duration. The infiltrate was found to be more widespread and to contain polymorphonuclear cells which were found in only one other monkey (case 13), in which they were very scanty and the pial reaction was focal and slight. In all other instances, the cells consisted of lymphocytic and mononuclear types and were few in number. In the majority of cases there was no pial reaction whatever, so that in general meningitis may be said to have been insignificant or absent.

Perivascular Infiltration.—The perivascular lesions of the group surviving from 41 to 84 days were far more marked and intense than those which were found in the cords of monkeys killed during the acute stage of the disease. According to Hurst, these infiltrates were most commonly localized about the large vessels in the neighborhood of the central canal, about the smaller vessels of the anterior and posterior horns, and about the vessels radiating through the white matter to the anterior fissure and to the periphery. In our series, on the other

hand, lesions of this type were most extensive in the anterior horns and about the vessels leading to and from the anterior fissure, while the smaller vessels of the posterior horns were less often and less seriously affected. The central zone usually remained uninvolved, unless a large lesion of the proximal portion of the anterior horn encroached on it. Radial lesions occurred in four or five cases, but consisted of only a single layer of cells in the perivascular space, except in case 4, in which the vessels were surrounded by a broad cellular sleeve.

In the anterior horns, large areas were frequently found in which the nerve cells had been destroyed, the architecture of the ground substance had been partly or totally obliterated, and perivascular infiltration and capillary proliferation were abundant (fig. 1).

The infiltrate varied in magnitude from a few cells in the Robin-Virchow space to a wide perivascular cuff, and at times extended beyond the adventitial space into the surrounding tissues. Throughout, the number of lymphocytes predominated over the mononuclear cells. Hurst estimated that 10 per cent of the perivascular cells in the acute stages of the disease were polymorphonuclear leukocytes. In the present series of chronic cases only a few cells of this type were observed in cases 1, 4, 5, 6, 7, 11 and 13. Specific stains were not made for plasma cells, but none were identified definitely in the routine preparations.

Tissue Infiltration.—Focal and diffuse infiltrations were more prominent in the earlier cases of this series than in the acute stage of poliomyelitis. Particularly in the areas of parenchymatous destruction of the anterior horns, many cells were seen lying about the periphery of the Robin-Virchow spaces, about the proliferating small vessels and about degenerating nerve cells, while independent cell masses were also common. The density of the infiltrate bore a direct relation to the extent of damage done to the tissues, so that lesions of decreasing severity were found in Clarke's column and in the posterior horns, in which they were often slight and focal in comparison with the dense generalized infiltration of the ventral portion of the cord. In contradistinction to Hurst's observations in acute poliomyelitis, the numerical cellular increase about the central canal was minimal, and the canal itself was invariably empty. In general, it may be said of

the gray matter that tissue infiltration varied directly with destruction of the neurons and inversely with the process of repair. Small focal lesions were also seen in the white matter together with a mild diffuse infiltration.

The cells taking part in this process were chiefly mononuclear, although a few polymorphonuclear leukocytes were found in cases 3, 5,

FIG. 1 (case 5).—Section of the lumbar cord showing an area of destruction in one anterior horn. Phosphotungstic acid and hematoxylin; × 33.

6 and 10, while case 1 again approximated the picture of acute poliomyelitis. Lymphocytes were plentiful in the more recent cases of the group, but rarer in those of long standing and in the absence of an extra-adventitial vascular process. They were at all times outnumbered by cells of the mononuclear type. No plasma cells were identified with the routine stains.

No attempt will be made to comment on the problem of the relation of the so-called "endothelial leukocytes" to the cells of the microglial series. The term "mononuclear" has been used to designate all

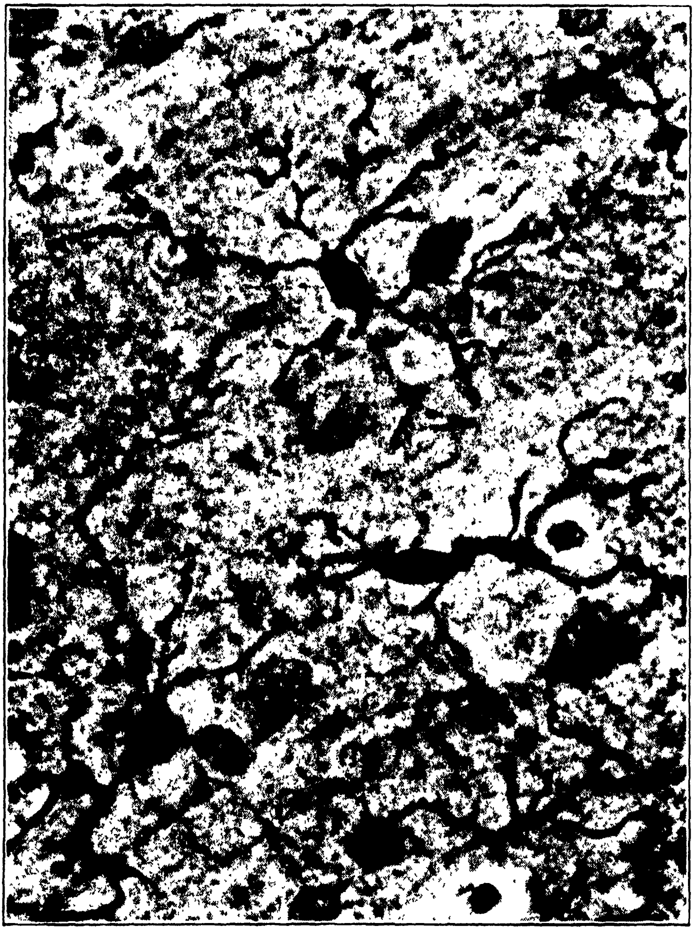


FIG. 2.—Normal microglia of the motor cortex. Silver carbonate Hortega; × 930. (Silver carbonate Hortega was used in staining all of the specimens appearing in figures 3 through 13, and 25 through 29.)

the cells of the tissue in filtrate other than polymorphonuclear leucocytes and lymphocytes. With the routine stains it was possible to differentiate the small, dark, elongated nuclei "Staebchenzellen,"

and the minute, round nuclei of oligodendroglia cells, which were often seen as satellites to the neurons of the group surviving from 242 to 309 days. In preparations other than gold and silver impregnations, it was not thought possible to distinguish with any degree of certainty the macroglia nuclei from those of the microglia showing pathologic changes.

The glial reaction was carefully studied in cases 4, 5, 6 and 8, while silver impregnations on formaldehyde fixed material were also available in cases 3, 7 and 10, together with gold sublimate preparations in

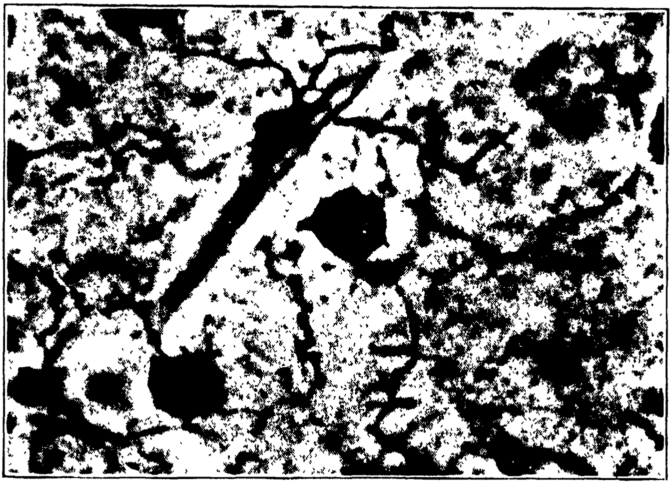
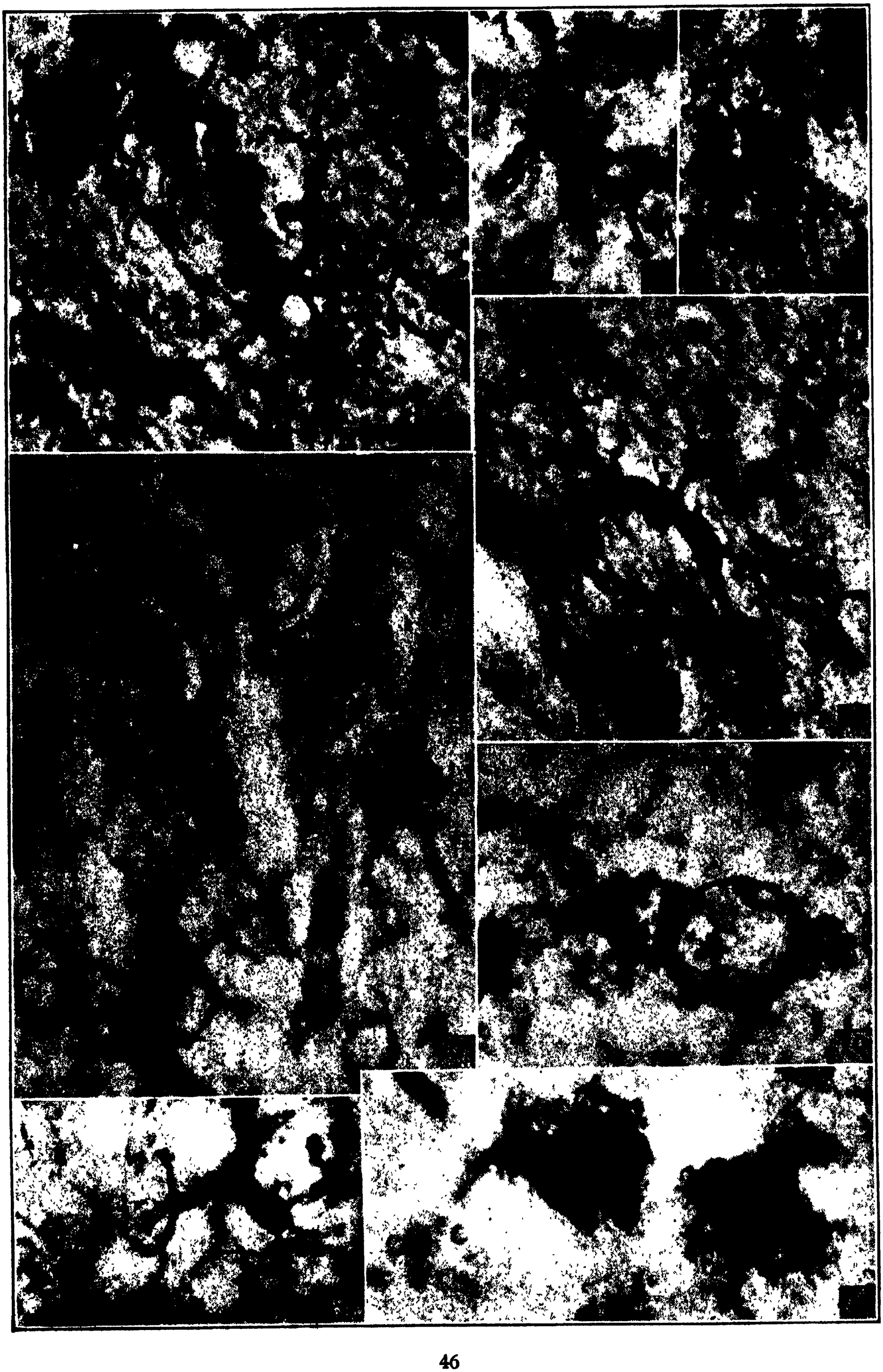


FIG. 3.—Normal oligodendroglia cells in the frontal cortex. Note the relative size of the microglia cells here and in figure 2; × 930.

cases 1, 3 and 10. There can be little doubt that the bulk of the focal infiltrate was composed of microglia cells. The changes from the normal microglia to compound granular corpuscles have been frequently and adequately described,[19] and are illustrated in figures from 4 to 11 inclusive. As a rule, mild damage to the nerve cells was accompanied by what might be termed pregitter cell changes in the microglia, whereas the destruction and disintegration of neurons called forth

19. Kubie, L. S., and Shults, G. M.: Forced Drainage of Cerebrospinal Fluid in Relation to Treatment of Infections of the Central Nervous System, Arch. Neurol. & Psychiat. 19: 997 (June) 1928. Hurst (footnote 7).



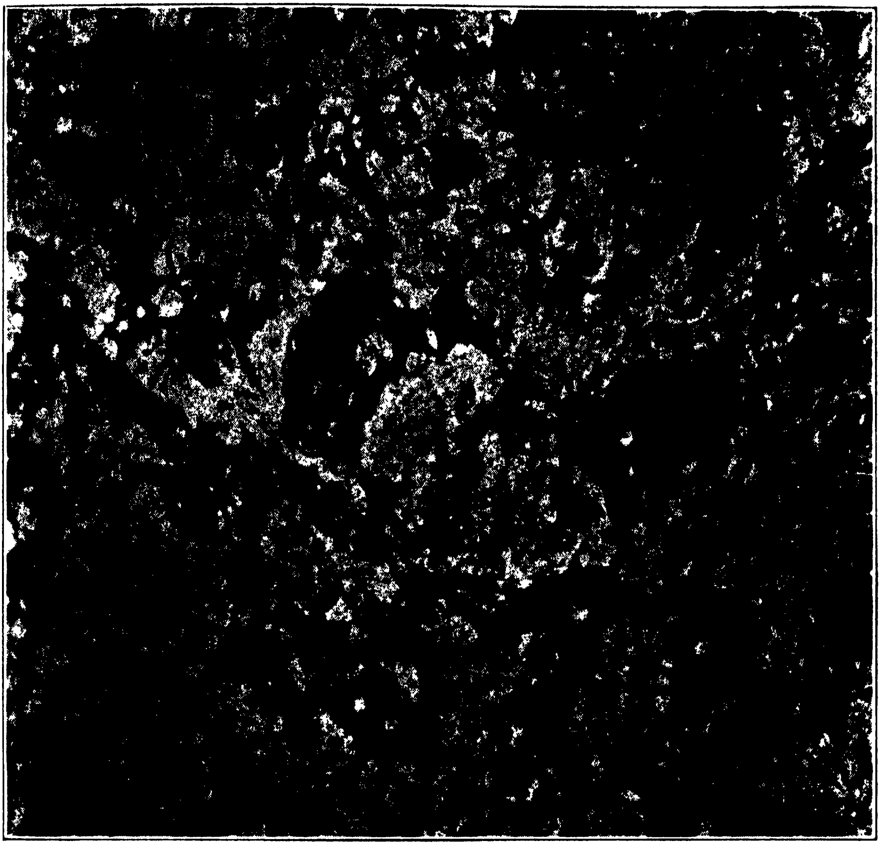


FIG. 12 (case 5).—Microglia showing pathologic changes in the area of destruction shown in figure 1; × 470.

FIG. 4 (case 5).—Microglia cell in the medulla, showing shortening and thickening of the processes. The oligodendroglia cells show no pathologic changes; × 930.

FIG. 5 (case 5).—Microglia cell in the medulla, showing enlargement of the cell body and shortening and clubbing of the processes; × 930.

FIGS. 6 and 7 (case 5).—Microglia cells in the medulla, with vacuolization of the cell body; × 930.

FIG. 8 (case 5).—"Staebchenzelle" in the medulla; × 930.

FIG. 9 (case 5).—Extensive vacuolization of a microglia cell (medulla); × 930.

FIG. 10 (case 6).—A typical gitter cell from the thoracic cord; × 930.

FIG. 11 (case 6).—Compound granular corpuscles laden with fat (lumbar cord). Several globules of lipoid are seen lying free in the tissues; × 930.

a response of granular corpuscles. These were rarely seen in cases in which only diffuse infiltration occurred. Since the lumbar region was more severely affected than the higher segments of the cord, it follows that the more advanced stages of microglial activity were more common there than in the cervical region (figs. 12 and 13). Parallel to this situation was the finding of abundant extracellular and intracellular fat in the anterior horns of the lower levels, while diminishing quantities were present in the thoracic and cervical cords of the group surviving from 19 to 72 days. We confirm Hurst's observation

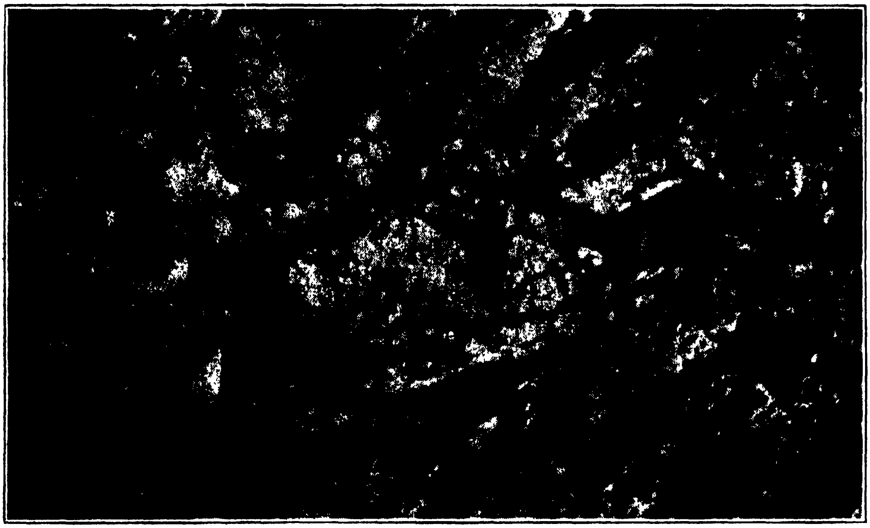


FIG. 13 (case 6).—Neuronophagia in the cervical cord. A group of phagocytic microglia cells are seen at the site of a degenerated nerve cell; × 470.

that, while many gitter cells were filled with fat, others showed large vacuoles containing no Fettpenceau-staining globules. The pregitter forms were also seen to contain fat in areas in which masses of bright red lipoid material were lying free in the tissues. In areas of milder activity, microglia cells carrying fat were observed only about and in the perivascular spaces. Free fat was also present both in that location and in the vascular lumina. No lipoid material was found in the gray matter of the cervical cords in cases 13 and 14.

The oligodendroglia cells, visible, although scanty, in the cross-

sections of the cords of the normal controls, were conspicuous by their absence in the monkeys with poliomyelitis, although an occasional cell of this type was seen in the posterior horns. It is probable that these rather delicate structures succumbed to the virulence of the infectious process in the earlier cases. Unfortunately, silver impregnations in the group surviving from 242 to 309 days were not available, so that the prominence of satellitosis about the nerve cells of the older animals could not be definitely confirmed as oligodendroglial, although the nuclei were apparently characteristic of these cells.

A comparison with normal tissues often revealed a generalized increase of both the fibrous and the protoplasmic astrocytes which was confined neither to the injured tracts nor to the areas of marked microglial reaction, although occasionally perivascular proliferation was seen. The cells themselves showed thick processes, frequently without secondary branches, heavy sucker feet and large, deeply impregnated cell bodies. Dividing cells were present, but rare. The areas of destruction showed a paucity of astrocytes consistent with the lack of fibrous replacement seen in the phosphotungstic acid preparations (fig. 1). In and near the periphery of these lesions the remaining cells were poorly stained and showed degrees of clasmatodendrosis varying from spirillar degeneration to fragmentation of the processes and feeble impregnation of the cell bodies (fig. 14).

Destruction of the Nerve Cells.—Case 1 presented the typical picture of damage to the neurons described by Hurst. The anterior horn cells were for the most part necrotic masses, which called forth a response of polymorphonuclear cells and microglial phagocytes, or else, in the stages of acute degeneration in which the cytoplasm was ragged in outline, acidophilic and structureless, while the nuclei were absent or eccentric and deformed, ill-defined or hypochromatic. These far out-numbered the second cellular type which was shrunken, containing hyperchromatic nuclei, and irregularly staining, often vacuolated cytoplasm in which no Nissl substance was distinguishable. These cells had been traumatized but were, in Hurst's terminology, "recoverable."

The degree of viability of these cells could be more definitely ascertained in the later stages in which acute degeneration was no longer present, but in which neuronophagia was prominent. In the group,

surviving from 40 to 84 days, as well as in that comprising cases of longer duration, the numerical cell loss was variable, not only from monkey to monkey, but also from level to level within the same spinal cord. In the lumbar region, the surviving nerve cells were fewer and more abnormal than in the cervical segments, the dorsal ranging between the two extremes (table 2). In the cases of intermediate duration, the shrunken cells were the predominating type and were char-

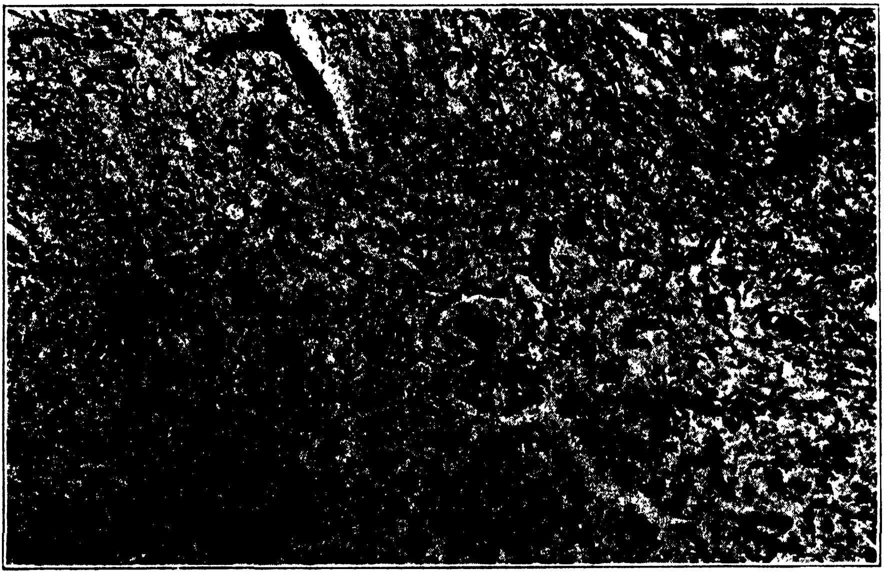


FIG. 14 (case 5).—Astrocyte proliferation in the area of destruction shown in figures 1 and 12. Clasmatodendrosis may be observed below and to the right of the vessel near the center of the field. The numerical increase of astrocytes in that location suggests that proliferation preceded degeneration. Cajal's gold sublimate; × 125.

acterized by hyperchromatism both of the cytoplasm and of the nucleus, by exaggeration of the processes, and by irregularity of contour or by their spindle shape (figs. 16 to 18). The Nissl substance was generally indistinguishable from the deeply staining cytoplasm, finely granular, or distributed about the cellular periphery. Nuclear detail was often similarly obliterated. Neurofibrils were occasionally visible at the proximal end of the processes, but, owing to the deep

black impregnation to which these cells were subject in the Bielschowsky preparations, they could rarely be traced beyond that point. When distinguishable, the fibrillar substance was granular and not clearly demarcated.

While many of these neurons were evidently injured but still viable, others, which had partially succumbed, were paler, more vacuolated and less regular in outline. The nuclei were on the whole better preserved than the cytoplasm. Microglial phagocytosis was frequently present. There was also a variable number of cells approximating the normal, generally near the periphery of the anterior horns.

FIG. 15.—Normal anterior horn cells of the lumbar cord. Phosphotungstic acid and hematoxylin; × 1,050.

In the group surviving from 242 to 309 days the numerical cellular decrease was greater in some instances, but the surviving cells showed few deviations from the normal other than slight distortions of contour and of the fibrillar network, which was sometimes knotted and clumped. In the proximal portions of the anterior horns, shrunken

cells of the type previously described were often seen (fig. 19). In contradistinction to the earlier cases, satellitosis rather than neuronophagia was a common observation (fig. 21).[20]

The same kind of destruction of the neurons, although in respectively diminishing degrees, was also present in the columns of Clarke, in the lateral horns and in the cells of the posterior horns. Although

FIG. 16 (case 5).—Section showing shrunken cells. Compare with similarly located anterior horn cells in figure 15. Phosphotungstic acid and hematoxylin; × 1,050.

these lesions may at times appear to be insignificant in comparison with the more dramatic process in the anterior horns, the disturbances of the sensory fiber tracts shown by the Weigert preparations, clearly demonstrate the degree of damage.

Figure 22 illustrates the characteristic picture of the cervical cords in which the outstanding lesion was that of the dorsal spinocerebellar

20. The term neuronophagia has been used in the narrower sense implying phagocytosis of damaged cells by microglial phagocytes, whereas satellitosis has been taken to signify the multiplication of oligodendroglia and microglia about apparently intact cells.

tract the fibers of which arise from the cells of Clarke's column. The tract of Goll remained intact, whereas degeneration of the fibers in the cornucommissural zone was seen in the areas which, in all probability, correspond to the neurons of association of cells which lie in the posterior horns. Similarly, in the lateral columns, injury to secondary neurons, the cells of which are located in the lateral portion of the anterior horns and at the base of the posterior horns, may be made responsible for damage to the lateral spinocerebellar and lateral spinothalamic tracts respectively. Only the dorsal spinocerebellar tracts

FIG. 17 (case 5).—Spindle cells in the anterior horn of the lumbar cord. Phloxine-methylene-blue; × 1,050

were fairly constantly involved, whereas a patchy distribution of the injury to the other pathways, including the lesions in the anterior columns, gave evidence to the irregularity of the disease process. It is possible that the lesions in the roof and the red nuclei may be made responsible for the injury to their spinal pathways.

The Fettpenceau stains showed little fat in the fiber tracts of the group surviving from 40 to 52 days, and although occasionally small focal areas in the lateral columns or diffuse droplets in the cornucommissural zone were seen, the dorsal cerebellar tracts remained strik-

ingly free from them. On the other hand, diffuse fatty degeneration was observed in the anterior and lateral columns and in the indirect posterior column pathways in cases 9 and 10, while cases 13 and 14 showed masses of fat in the dorsal cerebellar tracts.

The anterior roots of the lumbar cords were often infiltrated with fat, and the medullated fibers leading to them frequently showed degeneration. This occurred more rarely at the higher levels of the cord. No study of the peripheral nerves was undertaken.

Table 2 summarizes the perivascular, tissue, and nerve cell reactions according to an empirical scale ranging from mild to severe, according to the number of cells involved in the lesions, in order to compare the relative severity between levels and between cases. An estimate of this kind is necessarily inexact and is presented merely in the form of a key map to the preceding description.

Spinal Ganglia

Diffuse and focal lymphocytic infiltrates were present in the spinal ganglia in cases 4 to 6, and in cases 11 to 15 and were absent only in case 9. These, at times, replaced disintegrating ganglion cells. An increased number of capsular cells was probably more apparent than real, since the appearance of proliferation was often given by tangential sections (fig. 23). Microglial activity was not shown in case 4 on which silver impregnations were made and in which the lymphocytes were numerous.

Medulla and Pons

Here, as in the cord, pial infiltration was minimal and confined to a few cells about the anterior fissure and the nerve roots. At the level of the pyramidal decussation, the picture presented by the anteriorly situated motor nuclei was essentially that of the anterior horns at the lower levels, for extensive perivascular and tissue infiltrates, together with destruction of the nerve cells, were frequently present. The nuclei gracilis and cuneatus, on the other hand, were generally intact, and even when inflammatory lesions occurred in the posterior half of the medulla, only the mildest grade of disturbance of the neurons was observed.

At the higher bulbar levels this apparent preservation of the nerve

cells was even more remarkable in that, in the presence of frequent infiltrative inroads, the neurons showed only minor grades of degeneration, although glial response to these cells was the rule. In the group

TABLE 2

Spinal Cord

Case	Days	Lumbar			Thoracic			Cervical		
		Perivascular infiltration	Tissue infiltration	Degeneration of the nerve cells	Perivascular infiltration	Tissue infiltration	Degeneration of the nerve cells	Perivascular infiltration	Tissue infiltration	Degeneration of the nerve cells
1	19	Moderate	Severe	Severe	Mild	Moderate	Moderate	Mild	Severe	Severe
2	29	Mild	Mild	Mild	Mild	Mild	Moderate	Moderate	Moderate	Moderate
3	40	Moderate	Severe	Severe	Severe	Moderate	Moderate	Moderate	Severe	Moderate
4	41	Severe	Severe	Severe	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
5	47	Severe	Severe	Severe	Mild	Moderate	Moderate	None	Severe	Moderate
6	47	Moderate	Severe	Severe	None	Mild	Moderate	Mild	Severe	Moderate
7	48	Severe	Severe	Severe	Mild	Moderate	Moderate	None	Moderate	Moderate
8	52	Severe	Severe	Severe	Moderate	Severe	Severe	Mild	Moderate	Mild
9	59	Severe	Severe	Severe	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
10	72	Mild	Severe	Severe	Moderate	Severe	Severe	Mild	Moderate	Mild
11	84	Severe	Moderate	Moderate	Moderate	Mild	Moderate	Mild	Moderate	Mild
12	242	Mild	Moderate	Moderate	Mild	Mild	Mild	Mild	Moderate	Moderate
13	259	Moderate	Moderate	Moderate	None	Mild	Moderate	Moderate	Severe	Moderate
14	267	None	Mild	None	Mild	Mild	Mild	None	Mild	Mild
15	309	Mild	Moderate	Severe	None	Mild	Moderate	None	Mild	Moderate

surviving from 19 to 52 days, the perivascular lesions were at times so intense as to obliterate the nucleus of a cranial nerve totally or in part, so that, as in the cords, there were areas in which the parenchyma

was totally or partially replaced by the perivascular and tissue infiltrates, together with capillary proliferation, but even there the cells lying near the periphery of this zone were no more severely damaged than those at a little distance. Neurofibrillar stains were of little assistance in proving this apparent cellular integrity, as the impregnations were for the most part not entirely satisfactory or dependable.

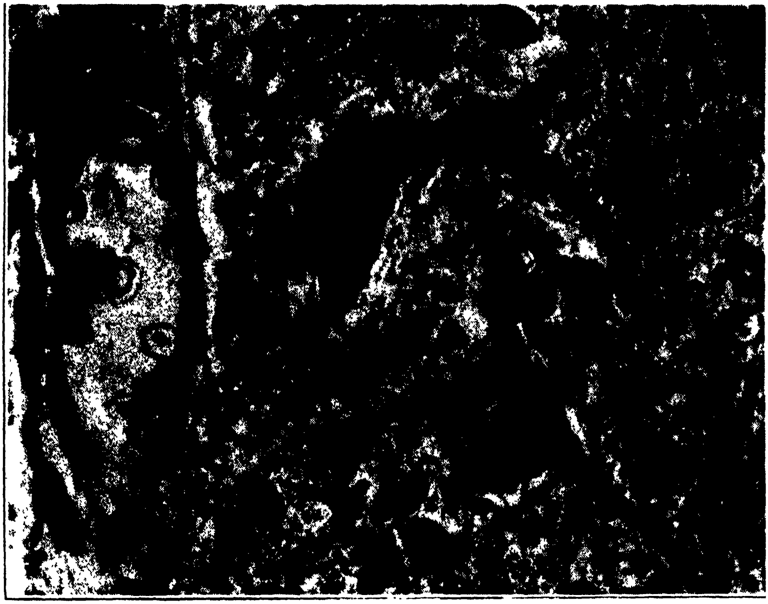


FIG. 18 (case 5).—Deformed nerve cells in the lumbar cord, showing sharply demarcated basophilic processes. No cellular structures other than the clumped Nissl substance of the upper cell are distinguishable. The nucleus of a mononuclear cell may be seen lying on each nerve cell. These should not be regarded as neuronophages in that they lie above the plane of the two neurons. Phloxine-methylene-blue; × 1,050.

No degeneration of the tracts or the fibers was seen in the Weigert preparations.

Since the perivascular lesions occurred most frequently near the mid-line, about the vessels of the anterior fissure, under the floor of the fourth ventricle, and near the angle of its floor with its lateral wall, the nuclei of the cranial nerves that suffered most severely were the

hypoglossal, the vestibular, Deiter's nucleus and the dorsal tenth. Lesions involving the twelfth nucleus often extended to the solitarius,

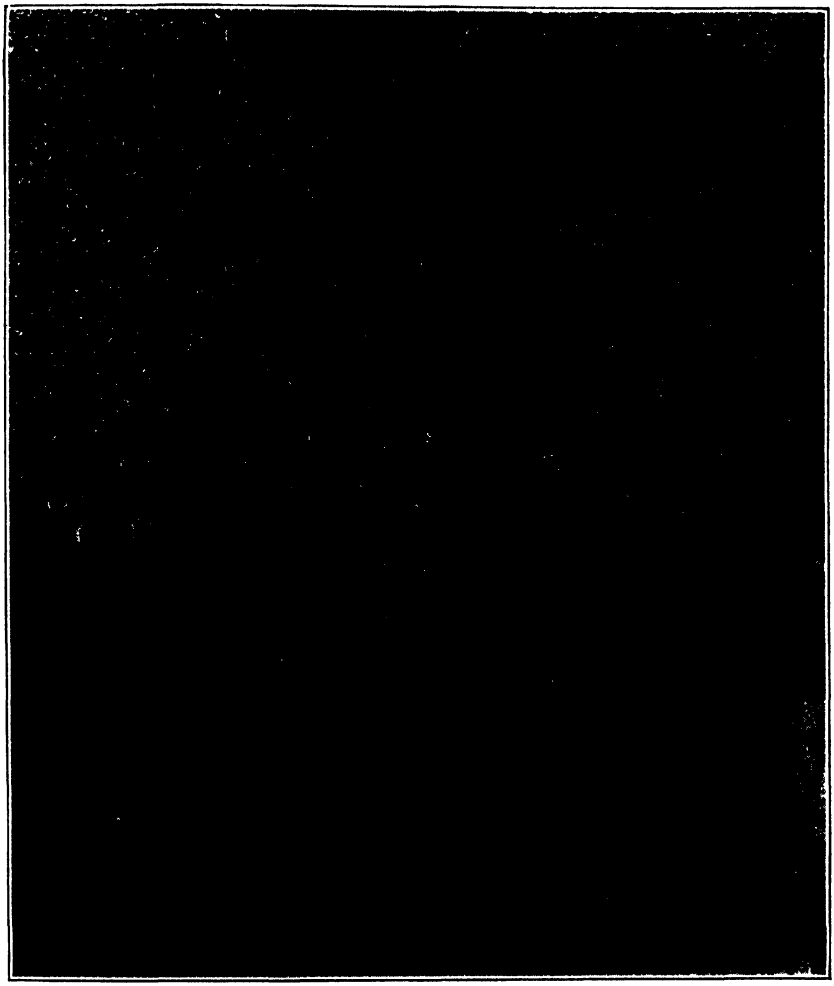


Fig. 19 (case 14).—The cervical cord of an animal that survived for 267 days after the onset of symptoms. There is no evidence of scar formation. Phloxine-methylene-blue; × 165.

and scattered patchy inroads on all the other nuclei of the cranial nerves were found at various levels in different monkeys, regardless of

their motor or sensory character, and dependent only on their casual location in the path of a perivascular lesion. The olives were often

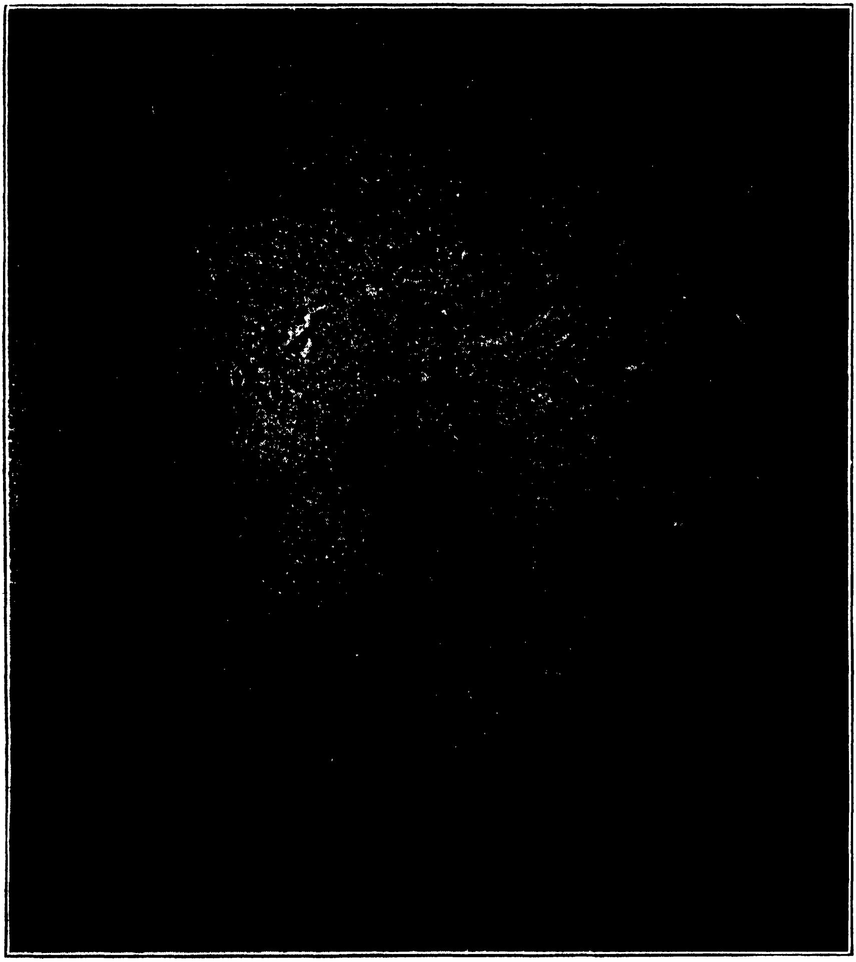


FIG. 20 (case 3).—The lumbar cord of an animal that survived for forty days after the onset of symptoms, showing extensive diffuse and focal tissue infiltration and a moderate perivascular reaction. Compare with figure 19 and table 1. Hematoxylin and eosin; × 165.

spared, although perivascular infiltrates were not uncommon in the substantia reticularis dorsal to them. Section cut through a higher

level of the medulla tended to show fewer lesions, so that the sixth nucleus was less often involved than the twelfth.

The reaction of the tissues was both diffuse and focal, the microglial activity varying with the intensity of the inflammatory process. Case 5 showed a generalized proliferation of astrocytes.

In the group surviving from 242 to 309 days, only longitudinal sections of the medulla were available, which could not be compared directly with the cross-sections in the other cases. Nevertheless, it was possible to ascertain that no perivascular lesions were present. In case 5 the glia nuclei about the apparently intact nerve cells showed a numerical increase over the normal.

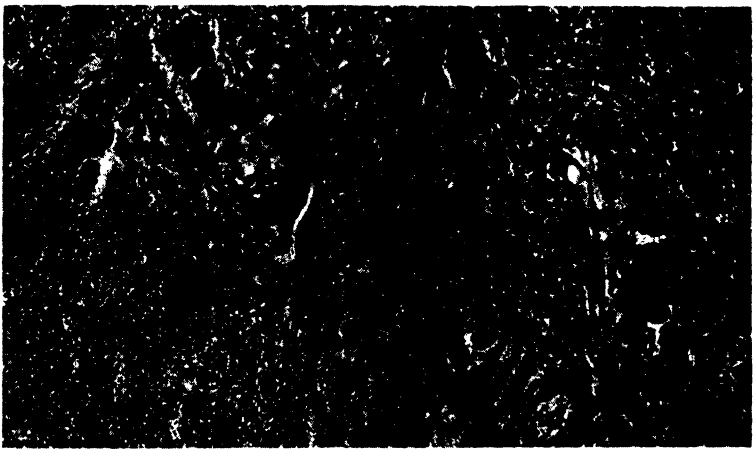


FIG. 21 (case 12).—Satellitosis in the lumbar cord of a monkey that survived for 242 days. Neuronophagia may also be seen about the two pale nerve cells lying near a deformed cell with four satellites. Phloxine-methylene-blue; × 440.

Midbrain

As the medulla, taken as a whole, showed less serious damage than the spinal cords, so the midbrain in cases 1, 3, 4, 5, 6, 7, 10 and 11 gave less evidence of inflammatory lesions than the lower brain stem. Hurst described the perivascular infiltration about the aqueduct in acute poliomyelitis as "more intense than at any other level." Large lesions of this character were seen about the iter in cases 1 and 3, but of the other seven monkeys only one (case 6) showed a mild perivascu-

lar reaction in that location. The red nucleus and the substantia nigra were extensively involved, while smaller perivascular lesions were found in the substantia reticularis near the midline and occasionally in the quadrigeminal plate.

Of the oculomotor and trochlear nuclei, and of the cells in the corpora quadrigemina, it is only possible to state that the cells showed mild changes in the face of infiltrates immediately adjacent to them, but that silver impregnations revealed marked proliferation of the microglia cells about them. Gitter forms were rare except in the red nucleus. Here the astrocytes showed clasmatodendrosis in the cases in which perivascular lesions caused advanced cellular destruction. It was characteristic of the patchy poliomyelitic invasion that occasionally one red nucleus was entirely obliterated while the other showed

TABLE 3

Incidence of Perivascular Lesions

Midbrain	Cases examined	Cases showing perivascular lesions
Corpora quadrigemina	8	5
Dorsal midbrain below aqueduct	7	6
Red nucleus	7	6
Substantia nigra	6	5

only the mildest changes. The cellular reaction corresponded to that in the anterior horns of the spinal cord. There were pale cells that had lost their stellate form and in which the cytoplasm was poorly defined while the nucleus was still distinctly visible. These were subject to neuronophagia of a type that heralded their ultimate removal. The second type of degeneration was represented by all the phases of shrinkage and hyperchromatism. Many cells were totally destroyed.

The status of the substantia nigra was similar to that of the red nuclei, although the cellular damage was usually not so great.

Basal Ganglia

The incidence of lesions in the basal ganglia, as shown in table 4, serves to demonstrate the relative immunity of the striate body as

compared to the globus pallidus and the thalamus. Perivascular lesions were less frequently present near the ventricular wall than in the neighborhood of the internal capsule, and it was in this region also that the pallidum was most frequently involved, although invariably to a lesser degree than the thalamus. The lesions in the putamen in case 4 were composed of only a few cells in the perivascular spaces.

Destruction of the nerve cells was never demonstrated with the routine stains, and only mild changes were noted, although tissue infiltration and neuronophagia accompanied the perivascular lesions. Silver impregnations in cases 4, 5, 6 and 8 showed advanced microglial activity in the thalamus and milder reactions of the same type in the globus pallidus. At times fat could be demonstrated about the perivascular spaces and in the vascular lumen, but cells containing fat were not seen in the tissues. The astrocytes in cases 5 and 6 gave evidence of clasmatodendrosis in the thalamus and showed no proliferation elsewhere.

Hypothalamus, Amygdaloid Nucleus and Geniculate Bodies

Large perivascular and infiltrative lesions were frequently seen in the lamina terminalis, in the hypothalamic region and in and near the amygdaloid nuclei. The geniculate bodies were studied in only a few cases, and in these no pathologic changes were found.

Cortex

The cortices of nine monkeys were examined. In cases 1, 3, 7 and 10 they were stained with hematoxylin and eosin. Celloidin sections had been cut through the whole brain so that not only both hemispheres but several sections through each lobe of the cerebrum could be studied. This afforded a good general picture, but was unsatisfactory from the point of view of detailed cellular structure. It was difficult to compare these with the thin paraffin preparations of the cortices in cases 4, 5, 6, 8 and 14 which were stained with phloxine-methylene-blue (methyl-thionine chloride, U. S. P.). Silver impregnations for microglia, oligodendroglia and neurofibrils were made of the cortices in cases 4 and 8, while all stains and impregnation were possible on

TABLE 4

Incidence of Infiltrative Lesions in the Basal Ganglia

Case		Caudatus	Putamen	Pallidum	Thalamus
1	Perivascular infiltration	+	+	+	+
	Tissue infiltration	+	+	+	+
2	Perivascular infiltration	0	0	+	+
	Tissue infiltration	0	0	+	+
3	Perivascular infiltration	0	0	0	+
	Tissue infiltration	0	0	0	+
4	Perivascular infiltration	0	+	+	+
	Tissue infiltration	0	+	+	+
5	Perivascular infiltration	0	0	0	+
	Tissue infiltration	0	0	0	+
6	Perivascular infiltration	0	0	..	+
	Tissue infiltration	0	+	+	+
7	Perivascular infiltration	0	0	0	0
	Tissue infiltration	0	0	0	0
8	Perivascular infiltration	0	0	..	..
	Tissue infiltration	0	0	..	..
9	Perivascular infiltration	0	0	0	0
	Tissue infiltration	0	0	0	0
10	Perivascular infiltration	0	0	+	+
	Tissue infiltration	0	0	+	+
11	Perivascular infiltration	0	0	0	+
	Tissue infiltration	0	0	0	+
12	Perivascular infiltration	0	0	0	0
	Tissue infiltration	0	0	0	0
13	Perivascular infiltration	0	0	..	..
	Tissue infiltration	0	0	..	..
14	Perivascular infiltration	0	0	..	+
	Tissue infiltration	0	0	..	+
15	Perivascular infiltration	0	0	0	+
	Tissue infiltration	0	0	0	+

two monkeys (5 and 6). In the second group of five cases, not more than one section through each cortical area was available. It follows that since the inroads of poliomyelitis are notoriously patchy in distribution, the following description of the pathologic changes may be considered somewhat fragmentary.

Pial Infiltration.—The meningeal reaction was slightly more marked

FIG. 22 (case 6).—Section of the cervical cord. The dorsal columns are spared except for the cornucommissural zone. The outstanding lesion is that of the dorsal spinocerebellar tracts, while diffuse degeneration may be seen in the lateral and anterior columns. Weigert-Kulschitsky; × 16.

over the cortices than over the spinal cords. Here again case 1 approximated the acute picture, for polymorphonuclear cells were seen whereas in the other cases the pial infiltrate was composed of lymphocytes and mononuclear cells. These were few in number and were usually found contiguous to vessels or in the depths of fissures. In the group surviving from 40 to 72 days, a slight leptomeningitic reaction was present over the motor and insular cortex of four monkeys,

over the occipital and temporal areas of three, and over two of the three frontal sections which were examined.

Perivascular Infiltration.—Although perivascular lesions were far

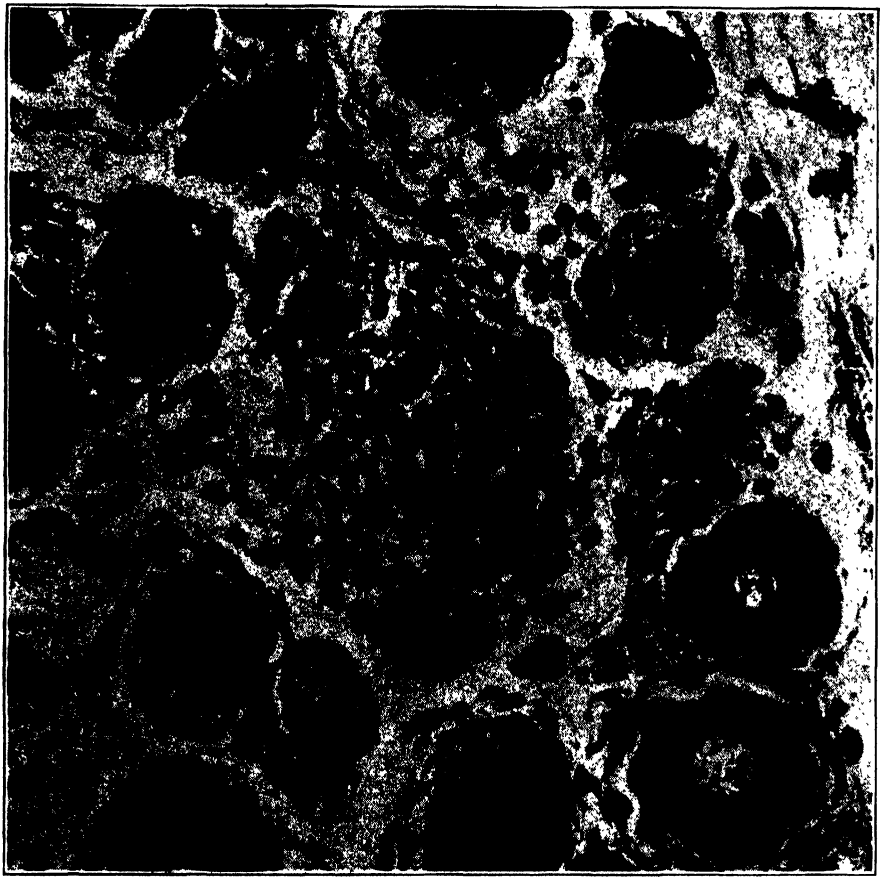


Fig. 23 (case 14).—Section of the spinal ganglion. A focus of lymphocytic infiltration overlying several disintegrating ganglion cells the capsule cells of which are still clearly visible. To the right a clump of capsular cells may be seen. Phosphotungstic acid and hematoxylin; × 440.

less prominent in the cortex than at the lower levels, case 1 showed extensive infiltration about the large vessels from the periphery and about the smaller ones in the deeper cortical layers. Here, too, cells

penetrated the perivascular spaces into the adjacent tissues. Of the eight other monkeys, only case 3 showed radial lesions in the motor area, while perivascular infiltrates of an extensive character were also seen in this monkey and in case 5. In general, the number of mononuclear cells and lymphocytes in the Robin-Virchow spaces formed only a thin cuff about the lumen of the smaller vessels.

Focal areas of destruction of the type seen in the spinal cords were rarely noted except in case 1. At times capillary proliferation was present in areas of deterioration of the nerve cells and glial infiltration, in which no perivascular lesions occurred.

Tissue Infiltration.—A degree of focal and diffuse tissue infiltration which was not approximated in any other instance was found in case 1,

TABLE 5

Incidence of Perivascular Lesions

Cortical area	Cases examined	Cases showing perivascular lesions
Anterior frontal	6	2
Motor area	8	6
Insula	7	3
Temporal	8	1
Occipital	7	1
Ammon's horn	8	1

in which many more lymphocytes were present than in the tissues from cases of longer standing. In general, the microglial proliferation bore a direct relationship to the damage to the nerve cells and to the perivascular reaction. There were few independent focal lesions. Gold sublimate impregnations were available only in cases of the group surviving from 40 to 52 days. In case 6 there was definite proliferation of astrocytes in the motor cortex (fig. 24) not unlike that described in dementia paralytica.[21] Comparison with the macroglia of the normal cortex in this area shows the glia cells to be more deeply impregnated and to possess coarser, shorter, frequently unbranched processes, and thick, long sucker feet. Dividing cells, but no astroblasts, were seen. Unfortunately, there were no preparations made from material

21. Ramón Cajal, S.: Ztschr. f. d. ges Neurol. u. Psychiat. 100: 738, 1926.

taken from the group surviving from 242 to 309 days, so that this reaction of the astrocytes could be traced no further.

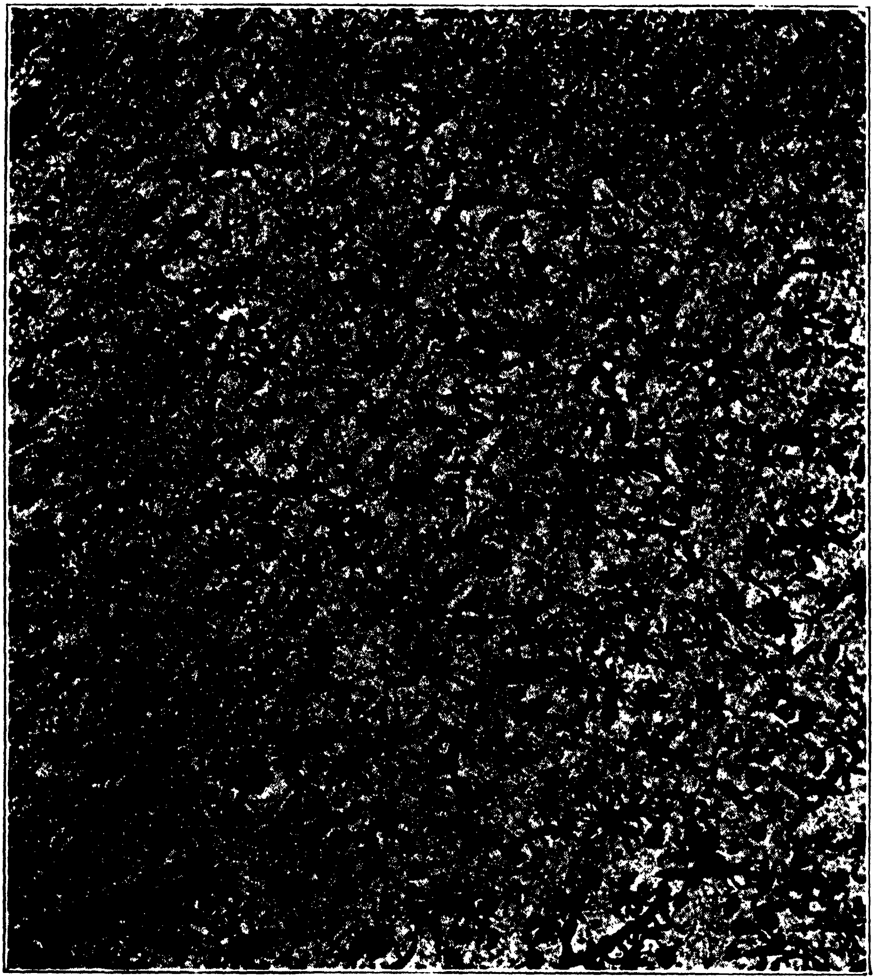


FIG. 24 (case 6).—An area of the precentral cortex showing hypertrophy and proliferation of the astrocytes; dividing cells may also be seen. Note the thick vascular attachments. Cajal's gold sublimate; × 220.

Due to the thickness of the hematoxylin and eosin sections, an accurate estimate of the tissue infiltration in cases 1, 3, 7 and 10 could

not be made, although it was possible to form a relative judgment on the cases as a whole, when comparison was made with similar material

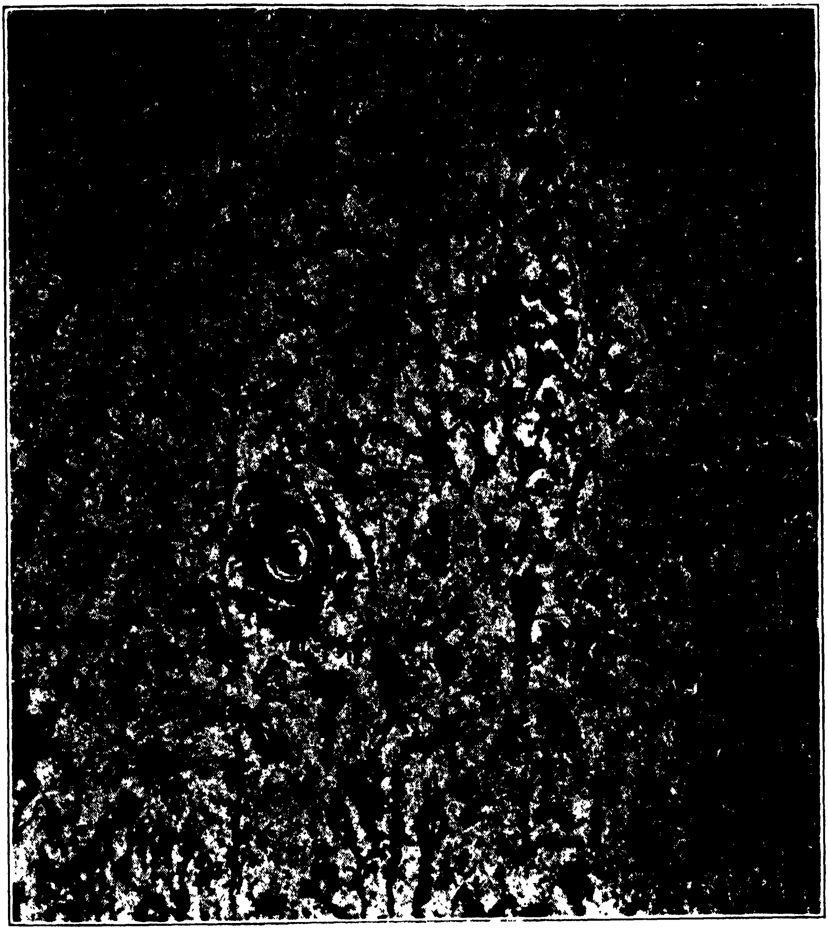


FIG. 25 (case 6).—Section showing the microglial reaction in precentral area of the cortex shown in figure 24. Above and to the right of the perivascular lesion is a focus of advanced destruction of the nerve cells and microglial proliferation. Below microglia cells may be seen lying about nerve cells and capillaries. The oligodendroglia shows no pathologic changes; × 220.

from normal monkeys. In cases 4, 5, 6 and 8, the cells shown by the phloxine-methylene-blue stain could be roughly correlated with the

silver impregnations in which the nerve cells were frequently visible also. It was found that while the damage to the neurons was frequently mild, judging by the routine stains, microglial proliferation about them was frequent and widespread, but silver impregnations of cortical areas which were extensively affected showed no compound granular corpuscles. Figures 25 and 26 illustrate the various pathologic microglia cells and their relation to degenerating neurons and proliferating capillaries. The section was cut from the same block as that in figure 24 and shows approximately the same cortical area as that in which astrocyte proliferation was demonstrated.

Swollen oligodendroglia were practically never seen. These cells were constantly and uniformly well defined in the sections stained by silver impregnation. In common with the microglia they were more heavily stained in the monkeys with poliomyelitis than in the normal controls, and while their number appeared to be somewhat increased in the cortex, this observation may have been more apparent than real, owing to their increased visibility (figs. 27 and 28). Such changes in the cytoplasm as were present were observed in the normal controls also.

TABLE 6

Incidence of Degeneration of the Nerve Cells

Cortical area	Cases examined	Cases showing degeneration of the nerve cells
Anterior frontal	5	5
Precentral and postcentral	8	7
Insula	7	2
Temporal	8	4
Occipital	6	4
Ammon's horn	7	4

Destruction of the Nerve Cells.—Degeneration of the nerve cells was more prominent in the anterior frontal, precentral and postcentral areas than elsewhere in the cortex, although no lobe remained consistently free from lesions. The approximate incidence of damage to the neurons, regardless of degree, is shown in table 6, which excludes case 1 on the ground that lesions were found in all areas throughout

the cortex, whereas the lobular distribution in the other cases was by no means uniform, and demonstrated the scattered character of the poliomyelitic inroads.

The fact that degeneration of the nerve cells bears no direct relationship to perivascular infiltration has frequently been discussed in connection with acute poliomyelitis, and was again demonstrated in the cortices of monkeys with the chronic disease. The architecture remained intact except for the rare perivascular and infiltrative encroachment on it. In the cortex as in the basal ganglia, it was striking that the cells at the periphery of the lesions frequently showed no more advanced changes than those at a distance. There appeared to be little specificity regarding the cellular layers attacked, and scattered areas of degeneration of the small neurons were seen here and there throughout the cerebrum. On the other hand, the Betz cells and the deeper strata of the motor cortex were affected consistently, and where lesions were present in Ammon's horn, they were often confined to the hippocampal gyrus.

The large motor neurons showed degeneration not unlike the milder forms of disturbance described in the anterior horns of the spinal cord. Pale cells showing loss of angularity, faintly staining cytoplasm and an eccentric but visible nucleus were in the minority. By far the more usual type were nerve cells that showed a tendency to shrinkage and staining abnormalities. Figure 31 represents a Betz cell in which the Nissl substance forms a thin marginal ring, and in which the cytoplasm stains a structureless reddish-purple merging into a bright basophilic patch at one pole. The nucleus is eccentric and the nucleolus is present, but the chromatin bodies are not clearly visible. The processes are faint and basophilic. In the hematoxylin and eosin preparations, cells of this general conformation frequently show a red nucleolus, and, where degeneration has proceeded a little further, a structureless red nucleus. In figure 32 a large motor neuron appears angular, shrunken, ragged and hyperchromatic. The Nissl substance is clumped, and the nuclear structure is no longer discernible. Another cell of the same type (fig. 33) has degenerated even further; it is deeply hyperchromatic and small, and approaches the spindle shape.

Similar changes occurred in the smaller cortical cells, although the more minute details were indistinguishable in the specimens

stained with the routine stains and in those from monkeys 5 and 6, stained with cresylecht violet. When stained with hematoxylin and eosin, the majority of cells which appeared to be pathologic were

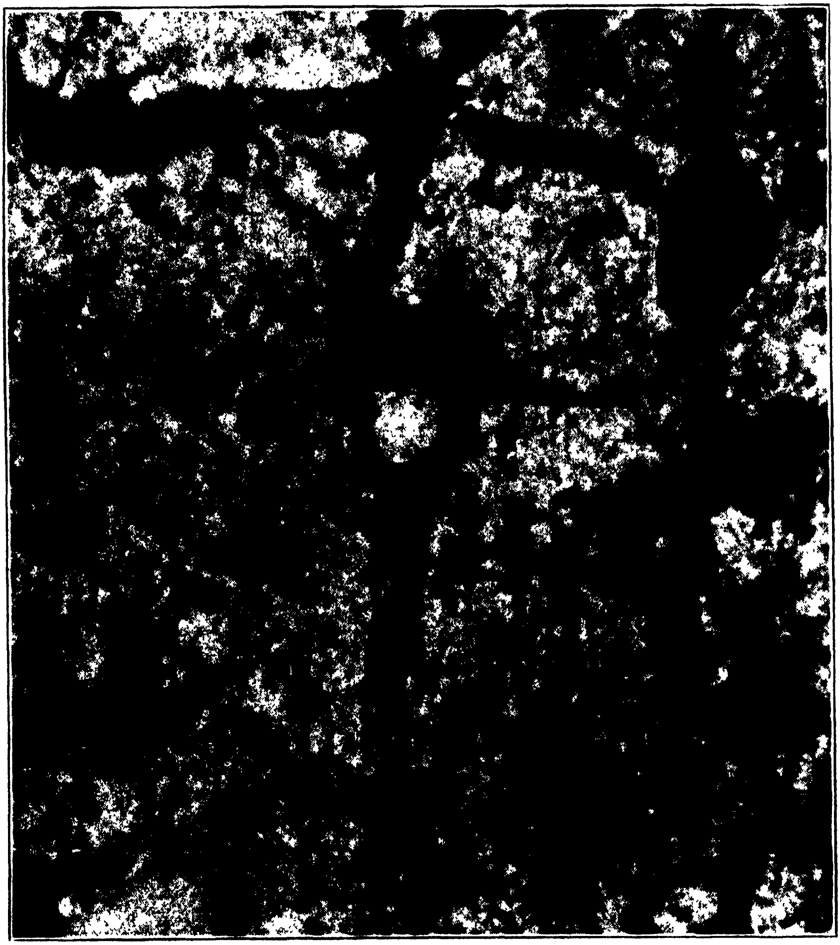


FIG. 26 (case 6).—Detail from figure 25. Note the microglia cells surrounding the cell body and axon, and lying on the capillary above the neuron; × 930.

spindle-shaped and showed basophilic nuclear degeneration and deeply acidophilic cytoplasm. In the phloxine-methylene-blue or phosphotungstic acid stains, the nuclei either remained clearly visible or be-

came uniformly and deeply purple (fig. 34). The smaller pyramidal cells often did not elicit a tissue reaction, but in other instances cells that were apparently not abnormal in other respects were surrounded by nuclei of small cells, the character of which could not be precisely determined with the routine stains, but which appeared to be microglia and oligodendroglia.

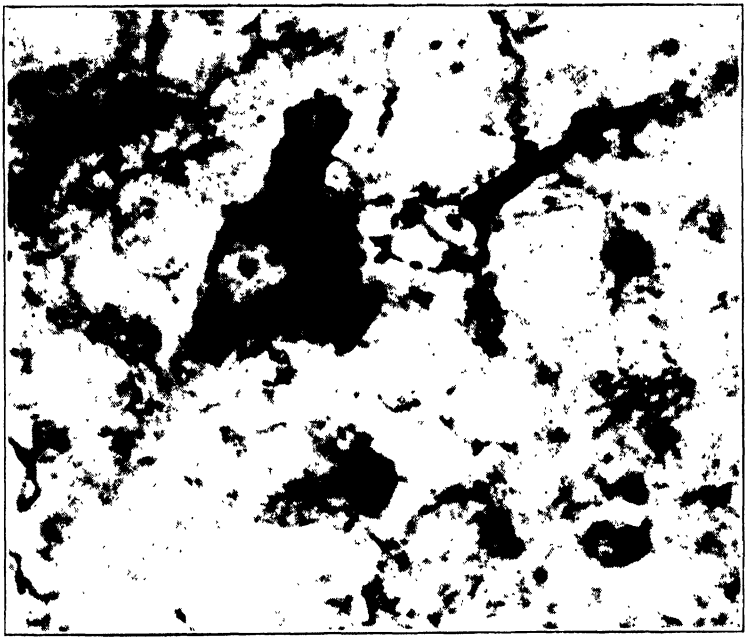


FIG. 27 (case 5).—A cell from the precentral cortex with two oligodendroglial satellites and one microglial neuronophage. The other two microglia cells may not be connected with the neuron; × 930.

As some of the staining reactions which were regarded as abnormal in the less severe cellular changes might have been due to technical errors, an attempt was made to impregnate the neurofibrils in cases 4, 5, 6 and 8. The results were variable both in the monkeys with poliomyelitis and in the normal controls, so that only such sections were considered reliable as showed an intact fibrillar system in the presumably normal cells while demonstrating various phases of neuro-

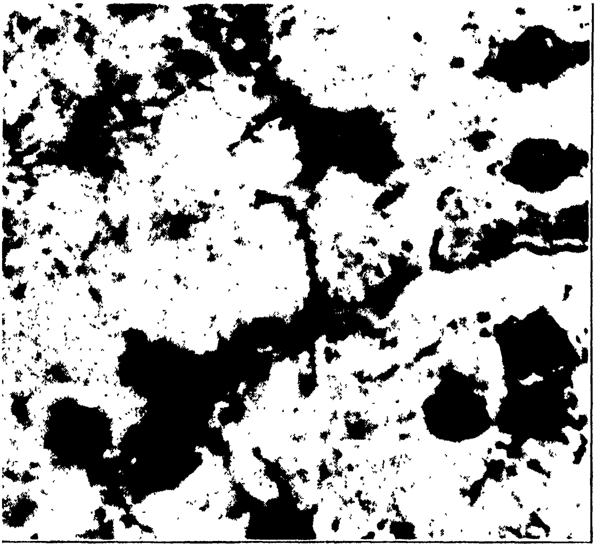


FIG. 28 (case 6).—Oligodendroglia cells on and about a capillary of the precentral cortex. A microglia cell showing pathologic changes is seen above the vessel; × 930.

FIG. 29 (case 6).—Microglia cells showing pathologic changes in an area adjacent to that shown in figure 28; × 930.

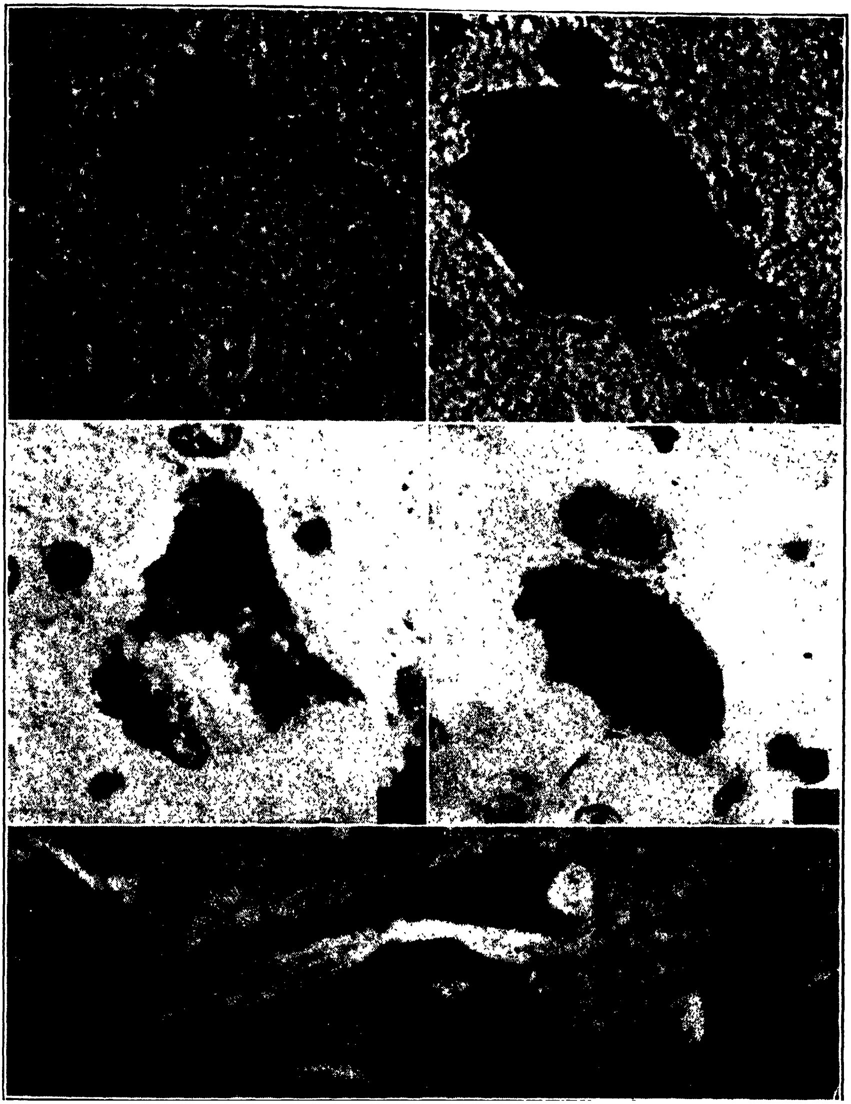


FIG. 30 (case 5).—Section of the precentral cortex showing a focus of mononuclear nuclei at the site of a cell that is distinguishable only by the projection of two faintly staining processes. Phloxine-methylene-blue; × 930.

FIGS. 31, 32 and 33 (case 5).—Section showing Betz cells described in the text. Phloxine-methylene-blue; × 930.

FIG. 34 (case 5).—Small pyramidal cells of the motor cortex showing shrinkage and hyperchromatism. Phosphotungstic acid and hematoxylin; × 930.

fibrillar degeneration in others. The axonal fibers showed disturbances more rarely than the intracellular network, which frequently appeared to be fragmented or destroyed. The evidence derived from this method failed to solve the problem of the number of cells affected in a given area, owing to the irregularity of the impregnation.

Cerebellum

The cerebella of nine monkeys were examined. Perivascular lesions were often seen in the restiform body, either spreading from the vestibular nuclei or independent of them. In the earlier cases, the roof and dentate nuclei were profusely infiltrated also, and their nerve cells were observed to be destroyed or in various stages of degeneration, although here too, many escaped damage entirely. The pericellular reaction was not marked, but focal infiltrative lesions were common, both in the nuclei and in the white matter. The Purkinje cells also showed staining abnormalities without calling forth a response from the tissue cells, but for the most part their neurofibrils appeared to be undisturbed.

COMMENT

It is not the purpose of this paper to discuss the route by means of which the virus penetrates into the nervous system, or the problems of the origin and significance of the cellular infiltrate, which have been well summarized by Hurst,[7] but rather to describe briefly the adequacy and inadequacy of the defense of the tissues against the onslaught of an infection. Unfortunately, this is an incomplete chapter in the history of the nervous system, since this description deals only with the battlefields and the early reconstruction period without stating precisely how far rehabilitation might ultimately be possible.

The period of early invasion was described by Hurst in his discussion of acute poliomyelitis.[7] Precisely what the function of the polymorphonuclear reaction to the nerve cells destroyed in the initial stages of the disease may be, remains unclear. In the light of their action as bacterial phagocytes elsewhere,[22] it is not impossible that these leukocytes bear a direct relation to the virus itself, although

22. Mudd, S.; Lucke, B.; McCutcheon, M., and Strumia, M.: Proc. New York Meeting Am. Soc. Path. & Bact., April 17–18, 1930.

there is no evidence in favor of this assumption a priori. It is questionable whether they take part in the process of neuronophagia, if the situation in the nervous system is analogous to that elsewhere in the body.[22]

Neuronophagia was observed in all its various forms in the cases of the intermediate group. It was evident that the microglia acted as the sanitation unit on the tissue battlefields in that the gitter cells performed the function of carrying off cellular débris, fat and detritus to the perivascular spaces or vessels. According to Penfield,[23] the compound granular corpuscles discharge the lipoid material directly into the blood stream.

The relationship of the microglia to the various types of degeneration of the nerve cells is only partly clear. It is probable that neuronophages do not attack intact cells, but in the basal ganglia and brain stem, certain cells appear to be unharmed except for the proliferation of microglia cells about them. Cortical cells, on the contrary, which were thought to show pathologic changes with the routine stains, called forth no reaction on the part of the tissues. Further, the less advanced forms of microglia were not seen to contain fat. As these were in the majority, except in the destructive focal lesions of the lower levels, it is not impossible that they have a function other than phagocytosis. Since the bulk of the tissue infiltrate diminished with the process of repair, it appears likely that this, if present, is of secondary importance.

As was already evident to Levaditi and Stanesco[10] in 1910, the inroads of poliomyelitis are not only irregular in distribution but also in phase. Active lesions were found in the medulla and basal ganglia in cases in which the cervical cord showed evidence of arrest and repair. In the latter stage satellitosis replaced neuronophagia. The cortical oligodendroglia cells also appeared to be slightly increased in number, which would suggest a reparative myelin reaction.[24]

Astrocyte proliferation of a generalized type was noted in the cord and cortex, but fibrous scar formation was never observed either in the phosphotungstic acid-hematoxylin or aniline blue preparations.

23. Penfield, W.: Am. J. Path. 1: 77, 1925.

24. del Rio Hortega, P.: Mem. r. Soc. españ. de hist. nat. 14: 5, 1928; abstr., Arch. Neurol. & Psychiat. 23: 557 (March) 1930.

In the areas of destruction, the parenchymatous structure was obliterated so that not only the nerve cells but also the astrocytes were destroyed, leaving a thin meshwork heavily infiltrated with microglia cells and lymphocytes. Repair in these areas was indicated only by capillary proliferation. How far scar formation had proceeded in the cases of long duration could not be determined in the absence of gold sublimate impregnations, but no such process was evident with the routine stains.

In regard to the perivascular reaction, the question arises whether the infiltrate may be regarded exclusively as a sign of active inflammatory activity. Doubtless the microglia cells derived from the membrana limitans gliae migrate from the vascular wall into the tissues,[7] but gitter cells laden with fat are frequently observed about the vessels as well as in the nervous parenchyma, which suggests that these cells are moving in the centrifugal current toward the spinal fluid or the blood vessels.[25] Similarly, the polymorphonuclear leukocytes that are seen in the perivascular spaces in all probability are being carried outward and eliminated, since the evidence is in favor of the theory that these cells reach the nerve tissue by penetrating the capillaries[7] and not the thicker wall of the larger vessels which they surround.

In general, the intensity of the perivascular infiltrate diminishes progressively from the cord to the cortex. It is true that frequently the medulla was more affected than the cervical cords, which showed a more advanced stage of repair. Mild lesions of this type are still present in the group surviving from 242 to 309 days. Monkey 15 had apparently made a good functional recovery, and yet signs of activity persisted in the lumbar cord and in the basal ganglia.

Destruction of the nerve cells was also most evident in the spinal cords. It has been shown both here and elsewhere[7] that the virus did not act specifically on motor cells, although it may be said of both the cord and the midbrain that the anteriorly situated nuclei were most intensely involved. On the other hand, the medullary damage to the nerve cells appeared to be regional, since the sensory and motor nuclei under the floor of the fourth ventricle were attacked with equal

25. Kubie, L. S., and Shults, G. M.: Bull. Johns Hopkins Hosp. 37: 91, 1925. Kubie (footnote 19).

severity, while in the basal ganglia the thalamus suffered more than the lenticular nucleus. At these subcortical levels degeneration of the neurons was frequently associated with perivascular lesions, and it was only in the cortex that, as in Hurst's acute cases, distinct disturbance occurred independently. Pathologic changes existed in the focal areas of motor or sensory cortex in which the vessels showed no inflammatory changes, although capillary increase was at times present, while neurons surrounding a perivascular cuff which did not penetrate the Robin-Virchow space often showed no more severe damage than those at a distance. The older writers referred to this type of degeneration, particularly to that of the precentral region, as "secondary." This term and its implications have been discussed by Spielmeyer[26] who pointed out that changes similar to those described in wallerian degeneration take place in many and various conditions. Were the virus carried by the blood stream, the assumption would be that penetration into the tissues occurred at the peripheral distribution of the vessel rather than along its course.

In conclusion, certain clinical application of these observations may be suggested. It is true that the infection in monkeys produced in the laboratory is far more severe than the disease in man, and it may be possible that the persistence of the lesions in human cases may be correspondingly less striking, but it seems highly probable that the process may continue to smolder long after the acute symptoms have disappeared, thus suggesting that prolonged rest may be indicated before reconstructive therapy is begun.

SUMMARY AND CONCLUSIONS

1. Histologic studies were made of the central nervous systems of fifteen *Macacus rhesus* monkeys surviving from 19 to 309 days after the onset of acute poliomyelitis. In all cases pathologic changes were seen.

2. The type of pathologic lesions found in the acute and reparative phases of poliomyelitis corresponded roughly to the duration of the disease.

26. Spielmeyer, W.: Histopathologie des Nervensystems, Berlin, Julius Springer, 1922, vol. 1, p. 263.

3. Inflammatory areas persisted in the central nervous systems of four animals which had made a good functional recovery.

4. A detailed study was made of the distribution and character of the lesions in the various levels of the nervous system, namely: spinal cord, spinal ganglia, medulla and pons, midbrain, basal ganglia, cortex and cerebellum.

5. These lesions have been interpreted as: (1) degenerative and inflammatory, including meningitis (negligible), perivascular and extra-adventitial infiltration, degeneration of the nerve cells and fiber tracts, and (2) reparative, including proliferation of microglia, astrocytes and capillaries.

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ZUR FRAGE DER QUANTITATIVEN BEDINGUNGEN BEI DER LIPOIDANTIKÖRPERBILDUNG DURCH KOMBINATIONSIMMUNISIERUNG

VON J. VAN DER SCHEER

(Aus den Laboratorien des Rockefeller Institute for Medical Research)

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In einer vor kurzem erschienenen Mitteilung gleichen Titels berichten F. Heimann und E. Weil[1]) darüber, daß sie bei der Immunisierung mit Lezithin oder alkoholischem Herzextrakt in Mischung mit Schweineserum Immunisierungseffekte auch dann erzielten, wenn sie das Serum in starker Verdünnung anwendeten. Wenn das Serum nicht stark verdünnt war, so war es möglich, die Menge des Organextraktes beträchtlich zu vermindern. Im Zusammenhang mit diesem Ergebnisse scheint es uns von einigem Interesse, über vor längerer Zeit ausgeführte Versuche kurz zu berichten, die auch die quantitativen Verhältnisse bei der Kombinationsimmunisierung zum Gegenstand hatten.

In einem Versuch, Kaninchen mit einer Mischung von Schweineserum und einem aus Pferdeniere hergestellten, Forssmansches Hapten enthaltenden Präparat zu immunisieren, war es aufgefallen, daß das Serum keines der fünf injizierten Tiere eine beträchtliche Steigerung des Hämolysintiters für Schafblut aufwies, obwohl das verwendete Präparat in vitro sehr wirksam war. Die Substanz war hergestellt wie früher beschrieben[2]). Die Tiere erhielten in Intervallen von einer Woche fünf intravenöse Injektionen, und zwar jedesmal 100 mg der Substanz aufgelöst in 5 ccm achtfach verdünntem Schweineserum. Nach fünf Injektionen gab das stärkste der Seren eine komplette Hämolyse nur bis zu einer Verdünnung von 1:25.

1) Zeitschr. f. Immunitätsf., Bd. 68, 1930, S. 403.

2) Journ. Immunol., Vol. 10, 1925, p. 732. Die dort erwähnten Präparate Pr. 2 und Pr. 3 wurden mit Alkohol ausgekocht, und der in Alkohol ungelöste, in Wasser leicht lösliche Teil wurde zu den Versuchen verwendet.

Der hämolytische Versuch wurde ausgeführt wie früher angegeben[1]; nur solche Tiere wurden zum Versuch genommen, deren Serum vor den Injektionen in einer Verdünnung von 1:25 keine komplette oder starke Hämolyse gab.

Ich dachte, daß dieses unerwartete Resultat möglicherweise auf die verhältnismäßig große Menge injizierter Substanz zurückzuführen sein könnte und wiederholte daher den Versuch im übrigen in gleicher Weise, nur nahm ich für jede Injektion 5 ccm zehnfach verdünntes Schweineserum, in dem diesesmal statt 100 mg nur 1 mg der haptenhaltigen Substanz gelöst war. In diesem Versuch wurden nach 5 Injektionen gut wirksame Seren erhalten: sie zeigten komplette Hämolyse bei vier Tieren bis zu einer Verdünnung der Seren von 1:1000, bei einem bis zu 1:500.

Um dieses Resultat zu bestätigen, wurde ein neuer Versuch gemacht, in dem parallel zwei Gruppen von je 5 Kaninchen injiziert wurden.

Die eine Gruppe erhielt wieder fünf intravenöse Injektionen von je 100 mg des Präparates gelöst in 5 ccm zehnfach verdünntem Schweineserum, die andere Gruppe 1 mg der Substanz in derselben Menge Schweineserum. Gleichzeitig wurde auch eine Gruppe von Tieren mit einer Kochsalzlösung der Substanz ohne Zusatz von Schweineserum injiziert (1 mg in 5 ccm Kochsalzlösung pro Injektion).

Wie in den früheren Versuchen wurden die Sera eine Woche nach der 5. Injektion abgenommen. Sie zeigten die folgenden Titerwerte (komplette Hämolyse, Ablesung nach einer Stunde, 37°):

	1 mg des Präparates in 5 ccm Kochsalzlösung					1 mg des Präparates in 5 ccm 1:10 Schweineserum					100 mg des Präparates in 5 ccm 1:10 Schweineserum				
Kaninchen No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Hämolysintiter (komplette Hämolyse) nach der 5. Injektion	25	100	25	25	100	500	500	500	250	2000	250	<25	<25	<25	100

Vor der Injektion gab das Serum keines der Tiere komplette oder starke Hämolyse in einer Verdünnung von 1:25. Nach diesen Resultaten dürfte kein Zweifel darüber bestehen, daß in dem vorliegenden Fall die Anwendung großer Mengen der spezifischen Substanz das

1) Journ. of Experimental Medicine, Vol. 38, 1923, p. 127.

Auftreten der Antikörper im Serum beeinträchtigte. Daß dieser Effekt so ausgesprochen ist, mag mit der starken Wirksamkeit des verwendeten Präparates in Zusammenhang stehen (Neutralisation gebildeter Antikörper?)[1]). Angaben über ähnliche Resultate liegen schon vor[2]) doch dürfte der beschriebene Fall ein besonders ausgeprägtes Beispiel darstellen.

ZUSAMMENFASSUNG

Immunisierungsversuche mit dem Hapten der Forssmanschen Substanz in Kombination mit Serum zeigten, daß durch Anwendung großer Mengen des Haptens der Immunisierungseffekt gehemmt wird.

1) Es wurde nicht untersucht, ob der Antikörpergehalt nach einem längeren Intervall eine Zunahme erfuhr.

2) Cf. E. T. H. Tsen: „Is there any quantitative relationship between antigen dose and antibody production?“ Journ. Med. Res., Vol. 37, 1918, p. 381. O. Schiemann, H. Loewenthal und H. Hackenthal, Zeitschr. f. Hyg., Bd. 112, 1931, S. 315.

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TRANSMISSION AND CULTIVATION EXPERIMENTS WITH HUMAN TRACHOMA AND THE EXPERIMENTAL DISEASE IN MONKEYS[1]

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In recent articles by Finnoff and Thygeson (1) and by Tilden and Tyler (2), an elaboration of the work of Noguchi (3) has been reported, namely, the recovery of *Bacterium granulosis* from cases of human trachoma in Denver (1) and Arizona (2), and the description of several additional cultural and biological characteristics of the microorganism. In this paper we shall present the results of cultivation experiments with conjunctival tissue from patients with trachoma residing in New York City, and of transmission tests in monkeys with the same material. The susceptibility of monkeys to conjunctival secretions from animals experimentally infected with trachomatous tissues or with cultures of *Bacterium granulosis* has also been studied. Thus the methods of Noguchi (3) have again been followed. The results of the repetition strengthen the view, first advanced by him, that *Bacterium granulosis* is closely related to human trachoma.

Transmission of Human Trachoma to the Monkey

The apparent failure of Noguchi to induce experimental infection in monkeys and apes by the use of human trachomatous tissue, in contrast to his success with cultures, may have been due to unfavorable experimental conditions. He made only two tests, inoculating material from American Indian cases of trachoma directly into a total of four *rhesus* monkeys and two apes. In the experiment with the latter, the tissues had been kept *in vitro* for 9 days before inoculation. The following tests show that conjunctival tissue from human trachoma in a white population, in New York City, induced in *Macacus rhesus*

[1] Preliminary reports of these experiments appeared in *Science*, 1929, **70**, 612; 1930, **71**, 263, 564.

monkeys characteristic granular conjunctivitis—a finding similar to that of Finnoff and Thygeson (1) with materials obtained from white patients in Denver.

Methods and Materials.—There were available for study nine patients residing in New York City, all having advanced trachoma of from 1 to 11 years' standing. With two exceptions (Cases 7 and 8), the patients exhibited scar tissue and characteristic pannus formation. The materials used consisted of the affected conjunctivae removed, for curative purposes, from the novocaine-anesthetized upper lid. We are greatly indebted to Dr. Martin Cohen[2] for these materials, and for other aid. The fresh tissue from each of eight of the cases was ground in saline solution and inoculated subconjunctivally into the left upper lid of two or three *Macacus rhesus* monkeys, using methods of injection already described (2, 3). In another series of tests, the conjunctival secretions from each of two patients were used as inocula. The excised conjunctiva of one of the latter patients was used for subconjunctival inoculation of monkeys, 19 days after the final collection of secretions. The secretions were taken on cotton swabs and, by gently rubbing the mucous membrane, were transferred directly to the conjunctivae of each of three monkeys. Nine swabbings at daily intervals were made with the secretions of the first case and seven with those of the second.

Only such monkeys as had smooth conjunctivae at the end of an isolation period of about 1 month were employed, so as to decrease the hazard of confusion with spontaneous folliculosis.

Results of Animal Inoculation.[3]—The results obtained after single subconjunctival inoculations of monkeys with suspensions of conjunctival tissue obtained from man will be considered first.

Case 1.—Male, 26 years old, Russian. Has had trachoma for 10 years. Scars and pannus present. Suspension of conjunctival tissue inoculated into the left conjunctiva of each of three *rhesus* monkeys (A, B, and C). All these animals showed the characteristic granular conjunctivitis described by Noguchi (3) as following inoculations of *Bacterium granulosis*. For example, Monkey A revealed follicles in the conjunctivae of both eyes on the 11th day after inoculation, and Monkeys B and C reacted similarly 4 days after the injection.

Case 2.—Female, 20 years old, Italian. Has had trachoma for several years. Scar tissue and pannus present. Monkeys D and E injected with suspension of patient's conjunctival tissue. Monkey D showed characteristic granular conjunctivitis in the left eye 33 days after the injection and in both eyes 41 days thereafter. Monkey E remained unaffected.

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[2] We are also grateful to Drs. Arnold Knapp, Ervin Torok, and Julius Wolff, all of New York City, for their cooperation.

[3] All operations were done under full ether anesthesia.

Case 3.—Male, 26 years old, American. Duration of trachoma, 7 or 8 years. Pannus and scars present. Monkeys F and G injected with tissue. Monkey F revealed the first signs of the characteristic experimental conjunctivitis in the left eye 10 days, and in the right, 45 days after inoculation. Monkey G failed to show lesions.

Case 4.—Male, 29 years old, German. Trachoma of uncertain duration, probably several years. Pannus and scars in left eye, scars only in right. Tissue from both upper lids pooled and injected into monkeys H and I. Monkey H developed the characteristic experimental disease in the left eye 7 days after inoculation. It died of tuberculosis, however, 1 month later. Monkey I showed granular conjunctivitis in both eyes also after 7 days.

Case 5.—Female, 50 years old, Russian. Duration of disease 10 years. Scar tissue and pannus present. The right and left upper conjunctivae were removed and a suspension of both tissues pooled was inoculated into the left conjunctiva of each of two monkeys, J and K, neither of which was affected by the injection.

Case 6.—Male, 48 years old, Italian. Duration of trachoma, 3 years. Scar tissue and pannus present. Tissue injected into two monkeys, L and M. Neither showed lesions.

Case 7.—Male, 31 years old, American. Has had trachoma for 11 years. 10 years ago, left tarsectomy performed and 5 years ago expression of follicles on left conjunctiva. The right upper conjunctiva showed large follicles, but no scars or pannus. A few follicles were also noted in the lower membrane and in the residual tissue of the left eye. Suspension of the upper right conjunctival tissue was inoculated into the left conjunctivae of each of two monkeys, N and O. Monkey N developed characteristic experimental conjunctivitis in both eyes 13 days after inoculation. Monkey O showed follicles in the left conjunctiva 6 days after injection, and in the right, 8 days later.

Case 8.—Male, 21 years old, German. Duration of disease 1 year. Treated for 2 months prior to tarsectomy. No scars or pannus present. Two monkeys, P and Q, inoculated subconjunctivally with suspension of patient's conjunctival tissue failed to become infected.

The next series of tests concern the production of the experimental disease in *Macacus rhesus* monkeys by means of swabbing the secretions of the patients on to the conjunctivae of the animals.

The first experiment was made with secretions obtained from Case 1, already mentioned, the secretions having been collected 19 days before the tarsectomy was performed. 13 days after the ninth and last swabbing, Monkey R developed the characteristic granular conjunctivitis; Monkey S died of tuberculosis during the swabbings; and Monkey T was unaffected.

The secretions for the second test were obtained from Case 9.

Case 9.—Male, 28 years old, Italian. Had trachoma for 2 years. Scars and

pannus present. 13 days after the seventh and last swabbing with this patient's secretions, three monkeys (U, V, and W) presented typical granulomatous changes in their conjunctivae. In none of the three animals, however, was extension of the affection noted in the untreated conjunctiva.

To summarize, a characteristic infection was produced in *Macacus rhesus* monkeys, either by means of a single subconjunctival injection of suspensions of conjunctival tissue from human trachoma, or by means of conveying the patient's secretions with cotton swabs to the animal's conjunctiva. By the former method, five out of eight patients' tis-

TABLE I

Record of Transmission Experiments

Case No.	Inoculation of material*		Recovery of *B. granulosis* from patient	Inoculation of *B. granulosis* into monkeys	Recovery of *B. granulosis* from animals inoculated with human material
	Subconjunctival	Swab			
1	+++	+−	+	+	+
2	+−		−	Not recovered	−
3	+−		−	" "	+
4	++		+	+	No experiment
5	−−		+	+	" "
6	−−		−	Not recovered	" "
7	++		+	+	+
8	−−		−	Not recovered	No experiment
9		+++	No experiment	No experiment	−

* Plus and minus signs in the first two columns represent for each sign the reaction of an individual animal.

sues yielded successful transfers to monkeys, whereas by the latter procedure, the swabbing of two patients' secretions induced granular conjunctivitis in animals.

The Experimental Disease.—The clinical appearance of the infected monkeys' conjunctivae was identical with that in the experimental disease induced by cultures of *Bacterium granulosis*. As described by Noguchi (3), the reaction consisted of a slowly progressing granular conjunctivitis, occurring first in the inoculated conjunctiva, and later spreading to the uninoculated membrane of both eyes. The outstanding features of the affection were congestion, edema, and thickening of the conjunctiva which was studded with numerous follicles,

covering the tarsal plates as well as the retrotarsal membrane. In the lower conjunctiva, similar but less marked changes occurred. In general, the disease in monkeys closely resembled the early stages of trachoma in man. In another article (4) we have alluded to the similarity of the clinical appearance of the experimental disease to Type I and II*a* (MacCallan's (5) classification) of human trachoma, and we have shown that by the superimposition of secondary infections with ordinary bacteria, the condition in the monkeys can be changed so as to resemble the florid stage of human trachoma. Indeed, the monkeys employed for the latter experiments were selected from among those described in this paper.

The microscopic changes in the conjunctiva removed from the experimental animals resembled those found by Noguchi (3) and ourselves in tissues inoculated with cultures of *Bacterium granulosis*. Moreover, the histopathological lesions were similar to those of human trachoma. They consisted chiefly of numerous large, typical lymphoid follicles, scattered monocytic infiltration, and thinning out or complete denudation of the epithelial layer. In addition, there was sparse scar tissue formation, especially in the subepithelial tissue and in a narrow zone around some follicles.

Recovery of Bacterium granulosis from Human and Monkey Lesions.—The tissue obtained from one patient (Case 9) was insufficient in amount for cultivation tests, hence cultures were made with material from only eight of the patients. The material obtained from four (Cases 1, 4, 5, and 7) yielded growths of *Bacterium granulosis*. In addition, the microorganism was recovered from two other patients whose tissues were not employed for transmission experiments. All of the cultures obtained were shown to be pathogenic for monkeys: in each instance characteristic granular conjunctivitis was induced. In view of the fact that the patients had advanced trachoma with a considerable degree of secondary infection by ordinary bacteria and that the disease had been of long standing, the numerical results of cultivation are what one might expect.

Cultures of *Bacterium granulosis* were also obtained from the conjunctivae of the following monkeys which were infected with the tissues or secretions of the patients: Monkeys A and C, Case 1; Monkey F, inoculated with tissue from Case 3, and Monkey N, with that from

Case 7. Table I summarizes the results of the transmission experiments.

We have shown, therefore, that affected tissue from human trachoma, as it exists in white patients in New York City, induces in *Macacus rhesus* monkeys characteristic granular conjunctivitis. We have also found that *Bacterium granulosis* can be isolated from the same trachomatous tissues used for successful inoculation of monkeys and from animals thus infected.

These positive results with New York cases of trachoma, when taken with those with white patients in Denver and Chicago, show clearly that *Bacterium granulosis* is not peculiar to the trachoma of American Indians in Arizona. In this connection, the isolation of the same organism from natives of Russia, Italy, and Tunisia should also be stressed (6).

Transmission Experiments with Monkeys' Conjunctival Tissue and with Cultures of Bacterium granulosis

In planning the following experiments on transmission of the experimental granular conjunctivitis induced by inoculation either of human trachomatous tissues or of cultures of *Bacterium granulosis*, an effort was made to imitate the way in which the incitant of trachoma might be implanted on the conjunctiva of man, namely, by contact or by rubbing the eyelids (7). The first experiment concerned the conveyance of infection by simple contact.

Conveyance of Infection by Contact.—The source of infection in this test consisted of two monkeys (A1 and B1). Monkey A1 had been originally inoculated subconjunctivally with *Bacterium granulosis* (Albuquerque strain No. 1), isolated by Noguchi (3) in 1926, on Feb. 10, 1928. Monkey B1 had also been inoculated with the same strain derived from a monkey passage (1928), on May 17, 1929. In both cases characteristic progressive chronic granular conjunctivitis resulted and on Nov. 8, 1929, when the contact test was begun, the conjunctival lesions were well marked. Two monkeys (C1 and D1), having smooth conjunctivae, were then placed in the same cage with Monkeys A1 and B1. They were examined on Nov. 26, 1929 (18 days later) and both showed definite signs of characteristic granular conjunctivitis in both eyes. In the case of Monkey C1, the congestion, edema, and follicles persisted for 198 days. Thereafter the conjunctiva healed. The condition in Monkey D1 is still present without any sign of amelioration (at the time of writing, the disease has endured for about 15 months). On Feb. 13, 1930, 79 days after the disease was established, a culture of *Bacterium granulosis* was recovered from the conjunctiva of this monkey.

The experiment was repeated. The sources of infection were two monkeys, originally inoculated successfully by the use of another strain of *Bacterium granulosis*. Two additional monkeys with smooth conjunctivae were caged together with the affected monkeys, and after 14 days' contact, the exposed animals revealed, in both eyes, characteristic granular conjunctivitis which is still persisting, now 10 months later. One of the monkeys infected by contact served for cultivation tests and *Bacterium granulosis* was recovered from its conjunctiva when the lesions were well advanced.

That contact can play a rôle in the extension of lesions has already been shown by Noguchi (3), who pointed out that lesions ultimately appear on the uninoculated conjunctiva of the infected monkey. The foregoing experiments show that infection can be secured merely by caging together uninoculated and inoculated animals. Similar results of infection by contact were obtained by Finnoff and Thygeson (1).

Transfer of Infection by Instillation of Cultures or Tissues.—In this test an attempt was made to simulate in monkeys other conditions which might initiate infection in man. Cultures were instilled and the eyelids rubbed.

Suspensions of three Arizona strains (2) of *Bacterium granulosis* were pooled and instilled into the conjunctival sac of each of two monkeys having smooth conjunctivae. The suspensions were dropped into the sac on each of 7 consecutive days, and after about 5 days' rest, two additional, similar series of daily instillations for 8 days were given. In all, 23 doses were administered. The monkeys remained unaffected.

In contrast with this control experiment, demonstrating the innocuousness of merely instilling suspensions of cultures, is the following test. Two monkeys, both with smooth conjunctivae, received in their conjunctival sacs about 5 drops of a suspension of the same cultures used in the foregoing experiment. After each of eight daily instillations, the eyelids were gently massaged for about a minute. 5 days after the last instillation and massage of the eyelids, both monkeys revealed characteristic granular conjunctivitis which persisted until their death from tuberculosis 6 weeks later. They had been kept in the same cage.[4]

In contrast,again, with the control experiment is the following: Into the conjunctival sac of two macaques having smooth conjunctivae was instilled a suspen-

[4] We have not been able to find any effect of tuberculosis on experimental trachoma or *vice versa*. As many monkeys die of tuberculosis among isolated normal stock animals as amongst trachomatous monkeys. The longest duration of experimental trachoma without supervening tuberculosis is, in our experience, 4 years.

sion of affected monkey tissues. The suspension was prepared by scraping the conjunctival tissue from six monkeys showing granular conjunctivitis which had been induced by inoculation of *granulosis* cultures, and suspending the tissue particles, or scrapings, in saline solution. Seven daily instillations were given; and 24 days later, both animals revealed definite, progressive, granular conjunctivitis which, at the present time, 8 months after inoculation, still persists.

The repeated simple instillation of cultures of *Bacterium granulosis* into the conjunctival sac of normal monkeys did not appear to infect the animals. On the other hand, the daily instillation of cultures followed by gentle rubbing of the eyelids after each instillation induced characteristic granular conjunctivitis. In addition, suspensions of conjunctival tissue freshly removed from monkeys having *granulosis* conjunctivitis, instilled repeatedly into the conjunctival sac of normal animals, gave rise to the characteristic experimental disease. The findings made it seem likely that some injury, even so slight a one as that produced by rubbing the eyelids, is a requirement for infection by the microorganism.

Conveyance of Infection by Swabbing Monkey Secretions and Cultures.—The transfer of the experimental disease of monkeys to normal animals was now attempted by swabbing secretions on the conjunctivae, thus imitating the mode of conveyance of infection from man to monkey, already described. And following this, another test was made to determine if swabbing with cultures of *Bacterium granulosis* would induce the infection.

Test with Secretions.—The source of the secretions was a *Macacus rhesus* monkey inoculated subconjunctivally in Feb., 1928, with tissue from another monkey infected with Albuquerque No. 1 strain of *Bacterium granulosis*. The conjunctival lesions were advanced and had endured for 22 months when the secretions were taken for the test. The material was collected on cotton swabs and transferred directly, by rubbing, to the smooth upper and lower conjunctivae of both eyes of two *rhesus* monkeys (A2 and B2). After six such swabbings, over a period of 8 days, the animals had developed characteristic granular conjunctivitis in both eyes. In Monkey A2 the lesions were progressive and endured for 2½ months, after which the animal died from tuberculosis. In Monkey B2 the condition still persists, after 1 year. A culture of *Bacterium granulosis* was recovered from the animal 10½ months after the disease first appeared.

Test with Cultures.—The cultures employed were the three Arizona strains (2) previously used in the instillation experiment described above. Saline suspensions of pooled growths were swabbed in the manner already described, on the clear

conjunctivae of two monkeys. Monkey A3 was swabbed 30 times in three series of eight and one of six daily dosages, with a rest interval of 8 days between series. Monkey B3 received only the first series of eight daily swabbings. Both monkeys developed characteristic granular conjunctivitis 4 days after the last dosage. In both monkeys, the lesions are still present, now after 1 year. From Monkey A3 a culture of *Bacterium granulosis* was recovered in the 7th month of the disease. No cultivation test was made on Monkey B3.

Saline suspensions of uninoculated leptospira medium, such as were employed for growth of the cultures, proved innocuous when swabbed upon monkey conjunctivae in precisely the same way.

It is plain that experimental trachomatous conjunctivitis can be produced in *Macacus rhesus* monkeys by repeatedly swabbing the conjunctivae with secretions from animals having *granulosis* conjunctivitis in an advanced stage, or with cultures of *Bacterium granulosis*.

SUMMARY AND CONCLUSIONS

1. Conjunctival tissue derived from alien and native American white persons in New York City, having trachoma in an advanced stage, has been used successfully to induce in *Macacus rhesus* monkeys characteristic granular conjunctivitis. The transfer of infection was effected either by a single subconjunctival injection, or by repeated swabbing with conjunctival secretions.

2. Pathogenic strains of *Bacterium granulosis* have been recovered from the trachomatous tissues of six out of eleven patients. In addition, the organisms have been isolated from the monkeys infected with human material.

3. Repeated swabbing with secretions obtained from monkeys having experimental trachoma has given rise to characteristic granular conjunctivitis in normal animals. In addition, repeated instillations of suspensions of conjunctival tissue fragments derived from affected monkeys have led to characteristic infection of the conjunctivae of normal monkeys.

4. Contact infection occurs in monkeys, as it has long been known to occur in human beings; animals with smooth conjunctivae developing the experimental disease when merely caged with infected monkeys.

5. Repeated instillation of cultures followed by rubbing the eyelids will lead to the disease in monkeys, a method of transfer which indicates one manner in which the affection may be transmitted from

man to man. Yet another manner of producing the experimental condition is by repeated swabbing with cultures of *Bacterium granulosis*. Noguchi has already reported the successful outcome of the subconjunctival inoculation of cultures and the spread of the disease from an infected conjunctiva to the other eye of the same animal.

6. Tissues derived from cases of human trachoma or from monkeys having the experimental disease induce, on conjunctival inoculation of *Macacus rhesus* monkeys, the same clinical and pathological effects as do cultures of *Bacterium granulosis*. The conjunctival lesions closely resemble, in clinical appearance and in microscopic changes, those of the follicular stages of trachoma in man.

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SEROLOGICAL TESTS WITH THE BLOOD OF CAVIA PORCELLUS AND CAVIA RUFESCENS

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In order to gain further information on the inheritance of serological characters in the hybridization of species,[1] an attempt has been made to find an instance of fertile species hybrids where a serological differentiation of the parent types would be possible. A material answering these requirements consists of the two sorts of cavies mentioned in the title, the cross of which has been studied extensively by Detlefsen.[2] As established by this author, the F_1 females are fertile when mated with either of the parent species.

When the blood and serum of the two species were allowed to interact, no hemagglutination or hemolysis took place. It was not difficult, however, to obtain hemolytic sera by several intraperitoneal injections of 0.5 cc. washed blood of the wild Brazilian guinea pig into the common domestic guinea pig. An experiment with one of the most active immune sera gave the following results:

TABLE I

2 Drops Immune Serum, 1 Drop 2½% Suspension of Washed Blood; Tests Kept at 37°

Blood of											Reading after
10 common guinea	0	0	0	0	0	0	w	0	0	0	5 min.
pigs	0	0	0	0	0	0	d	0	0	0	1 hr.
5 hybrids	w	w	st	w	c						5 min.
	st	st	a.c	st	c						1 hr.
10 wild Brazilian	a.c	c	a.c	a.c	c	c	c	c	a.c	c	5 min.
guinea pigs	c	c	c	c	c	c	c	c	c	c	1 hr.

w = weak; d = distinct; st = strong; a.c = almost complete; c = complete hemolysis.

[1] Landsteiner, K., and Van der Scheer, J., *J. Immunol.*, 1924, **9**, 213, 221.

[2] Carnegie Institution of Washington, Publication No. 205, 1914.

In all, 20 Brazilian and 23 common guinea pigs were examined. Whereas the cells of all the former reacted positively with varying intensities, the blood of only one common guinea pig gave a distinctly positive reaction, although to a considerably lesser degree than any individual of the other species. The bloods of 6 hybrids (♂ *Cavia rufescens* × ♀ *Cavia porcellus*) showed positive reactions, but with one exception these were weaker than those obtained with the wild type.

On inactivating the sera, similar differences were demonstrable by hemagglutination.

It is proposed to extend these studies to further generations and to search for other reactions differentiating not only the blood cells but, if possible, also the proteins of the two kinds of animals.

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ATOMIC SCATTERING POWER OF COPPER AND OXYGEN IN CUPROUS OXIDE

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ABSTRACT

Atomic F-curves for the copper and oxygen in Cu_2O, have been obtained for $K\alpha$ radiation of copper from a measurement of the principal powder reflexions. The F-curves are compared with the F-curves for metallic copper and for oxygen in NiO and found to be identical.

Measurements of the reflecting power of cuprous oxide for the $K\alpha$ radiation of copper were made, in order to compare the atomic F_{Cu}-curve of the combined copper with that of metallic copper[1] and also that of oxygen with the F_{O}-curve of oxygen in NiO.[2]

The ionization spectrometer and high potential source used in this work have been described in previous publications.[3] A Siemens-Phoenix copper target tube operating at a potential of 30 KV served as the source of x-rays. The radiation was filtered with 0.001 inches of nickel foil in order to cut down the background scattering from the Cu_2O. The ionization current was measured with a Cambridge Compton electrometer having a sensitivity of 11 meters per volt (scale distance = 2 meters).

Samples were prepared by pressing into a suitable briquet, cuprous oxide which had been powdered until it would pass through a 325 mesh sieve. The surface of each briquet was filed to remove preferred orientation which might occur due to crystal grains coming in contact with the smooth metal of the press.

[1] A. H. Armstrong, Phys. Rev. **34,** 931 (1929).

[2] R. W. G. Wyckoff, Phys. Rev. **35,** 583 (1930).

[3] R. W. G. Wyckoff and A. H. Armstrong, Zeits. f. Krist. **72,** 319 (1929).

Two specimens of Cu_2O were used. Analyses for the copper content of the specimens showed for No. 1, 87.4 percent and No. 2, 86.0 percent of copper as compared with 88.8 percent for pure Cu_2O. The results given below are computed from measurements on specimen No. 1. A comparison of the reflexions from No. 1 and No. 2 showed no difference in intensity within the accuracy of the measurement, except for the (200) reflexion. The (200) reflexion for No. 2 is greater than that for No. 1, on the other hand the results indicate that No. 1 is greater than the correct value, and by approximately the same amount. These discrepancies are due to a small amount of CuO present, a strong reflexion of which occurs at about the same angle of reflexion as the (200) of Cu_2O. The amount of CuO present is not sufficient to reduce the intensity of the other reflexions by an amount that could be detected.

Measurements of the radiation reflected from a set of planes were made by taking intensity readings, at intervals of five minutes of ionization chamber angle, over the width of the line. (The slits limiting the beam entering the ionization chamber, subtended an angle of 9′.) These intensities were plotted against ionization chamber angle. Thus plotted the area under the resulting curve is proportional to the power of the reflected beam. The area under the curve for the (200) plane was taken equal to 100 units, the other reflexions being measured in these units. To place the measurements on a standard scale the (220) reflexion for NaCl was also measured in terms of the same units.

Since the power $P_{[hkl]}$ of the reflexion from a plane $[hkl]$ is given by[4]

$$P_{[hkl]} = \frac{1}{32\pi} \lambda^3 \frac{e^4}{m^2c^4} \frac{1}{r} \frac{\rho'}{\rho\mu} N^2F^2jP_0 \frac{1+\cos^2 2\theta}{\sin\theta \sin 2\theta}$$

if the relative power of reflexion P_s and the absolute F_s is known for NaCl (220) the desired F-values for Cu_2O may be calculated from

$$F^2_{[hkl]} = K \frac{P_{[hkl]}}{j} \frac{\sin\theta \sin 2\theta}{1+\cos^2 2\theta}$$

where

$$K = \frac{N_s^2}{N^2} \frac{\mu\rho}{\mu_s\rho_s} \frac{F_s^2 j_s}{P_s} \frac{1+\cos^2 2\theta}{\sin\theta_s \sin 2\theta_s}$$

[4] A. H. Compton, X-Ray and Electrons. (New York 1926) p. 131.

and N is the number of atoms per unit volume; j, the number of reflecting planes; θ, the angle of reflexion; μ, the absorption coefficient; ρ, the density; F, the structure factor; P, the power of reflexion. The subscript s refers to NaCl. Absolute F-values for NaCl have not been determined for copper radiation. There is evidence,[5] however, for the fact that if the radiation used is not too close to the characteristic radiation of an element the atomic F-curve as a function of $(\sin\theta/\lambda)$ is independent of the wave-length λ. On this assumption the structure factor $F_{[NaCl220]}$ has been assigned the same value as for the $K\alpha$ radiation of molybdenum, which has been found to be[6] $F = 62.48$.

Values for the absorption coefficient were found by using Jönsson's general formula.[7] The coefficients used are:

Material	μ	ρ
NaCl	161.3	2.16
Cu_2O	113.6	5.88

The greatest uncertainty in determining the absolute F-values probably lies in assigning values to the absorption coefficient.

Cuprous oxide has a cubic structure containing 2 molecules of Cu_2O to the unit cell. The oxygens are in a body centered cubic arrangement, with the copper atoms arranged in tetrahedra around the oxygen atoms. For this type of structure the structure factor $F_{[hkl]}$ for the plane $[hkl]$ is given by the following equation[8]

$$F_{[hkl]} = F_{O}\left(1 + e^{\pi i(h+k+l)}\right) + F_{Cu}\left(e^{\pi i/2(h+k+l)} + e^{\pi i/2(h-k-l)} + e^{\pi i/2(-h-k+l)} + e^{\pi i/2(-h+k-l)}\right)$$

where F_O and F_{Cu} are the atomic scattering factors for oxygen and copper. By means of this expression the relation between F_O and F_{Cu}, which are to be determined, and the structure factor F can be found for any reflexion.

[5] E. Wagner and H. Kulenkampf, Ann. d. Physik **68**, 369 (1922).

[6] R. W. James and E. M. Firth, Proc. Roy. Soc. **A117**, 62 (1927).

[7] E. Jönsson, Uppsala Univers. Arsskrift (1928).

[8] H. Ott, Handbuch der Expermentalphysik. Vol. VII, part 2. (Leipzig 1928) p. 270.

Experimental Results

Table I gives the measured values of relative intensity, the structure factor F as determined from them, and the value of F in terms of F_O and F_{Cu}.

TABLE I

Plane	$(\sin\theta)/\lambda$	Int.	F	Structure factor F:
110	0.164	17.1	11.7	$2F_O$
111	.202	288.	73.3	$4F_{Cu}$
200	.233	100.	58.9	$4F_{Cu} - 2F_O$
220	.331	85.0	58.6	$4F_{Cu} + 2F_O$
311	.388	68.3	44.8	$4F_{Cu}$
222	.406	14.1	36.8	$4F_{Cu} - 2F_O$
NaCl 220		69.0	62.48	$(4F_{Na} + 4F_{Cl})$

From these values of F the best values for F_{Cu} and F_O are found by graphically constructing smooth curves of $4F_{Cu} + 2F_O$, $4F_{Cu} - 2F_O$, F_{Cu} and F_O against $(\sin\theta/\lambda)$ such that the measured point will lie on these curves.

Table II gives the values of F_{Cu} and F_O thus determined:

TABLE II

$(\sin\theta)/\lambda$	F_O	F_{Cu}	F(determined from F_O and F_{Cu})
0.104	5.9		11.8
.202	5.5	18.3	73.2
.233	(5.0)	(16.4)	(55.6)*
.331	3.4	12.9	58.4
.388	2.6	11.2	44.8
.406	2.4	10.4	36.8

* This value is 6 percent lower than the measured value of F_{200}. The presence of CuO in the sample is responsible for the measured F being too great.

Fig. 1 shows graphically a comparison between the values of F_{Cu} for Cu_2O and metallic copper. F_{Cu} for Cu_2O is shown as a full line, and black circles, and F_{Cu} from copper metal[1] as crosses. The F curves being plotted against $(\sin\theta/\lambda)$.

Fig. 2 gives the same comparison for F_O from Cu_2O and NiO. The full curve and black circles showing the F curves for oxygen in Cu_2O. The crosses that for oxygen[2] in NiO.

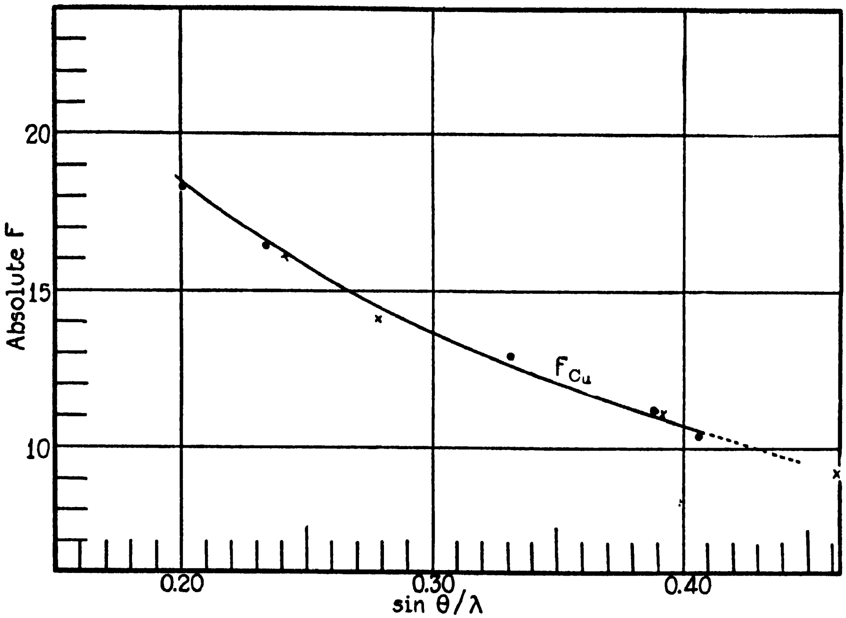

FIG. 1. The *F*-curves of copper atoms from Cu_2O and metallic copper

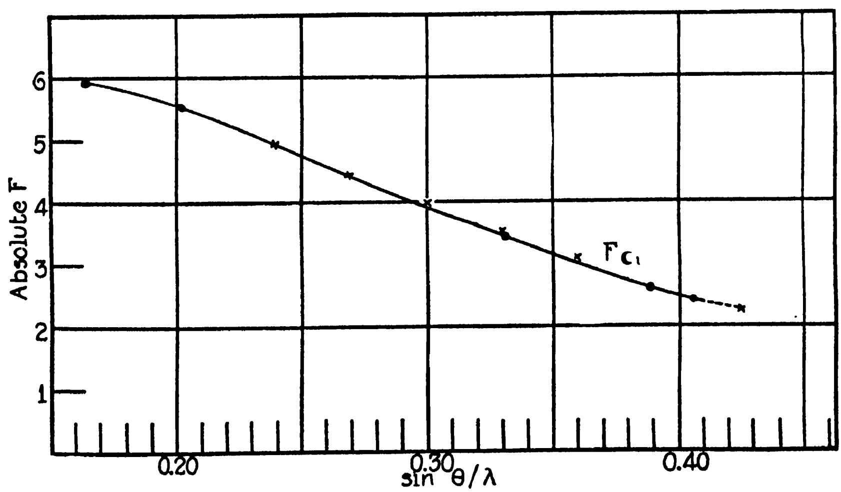

FIG. 2. The *F*-curves of oxygen atoms from Cu_2O and NiO

Both the F_{Cu}-curve and F_O-curve are within the limits of the accuracy of the determination, the same for Cu_2O as for metallic copper and NiO.

In conclusion I wish to thank Dr. R. W. G. Wyckoff to whom I am indebted for the use of his powder spectrometer, and for invaluable aid and advice in carrying out this work.

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ON THE MONOMETHYL GLUCOSE OF PACSU

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For an investigation now in progress in this laboratory, 4-methyl glucose was required, and as Pacsu[1] had prepared a substance to which he ascribed this structure, we undertook its preparation by his procedure. However, in a recent paper, Brigl and Schinle[2] describe 2-methyl glucose with physical properties practically identical with those given by Pacsu for his methyl glucose. Moreover the 2-methyl-1,1-diethylmercapto-*d*-glucose of Brigl and Schinle is apparently identical with the methyl-1,1-diethylmercapto-*d*-glucose which resulted when we extended Pacsu's procedure to diethylmercaptoglucose. These considerations led us to subject the methyl glucose of Pacsu to more rigorous tests with the result that its identity with the 2-methyl-*d*-glucose of Brigl and Schinle has been definitely established.

The methyl glucose was prepared from 1,1-dibenzylmercaptoglucose according to the procedures of Pacsu. The analysis corresponded to a monomethyl hexose, and the melting point and rotations agreed with those reported by Pacsu, as well as with those reported by Brigl and Schinle.

On treatment with phenylhydrazine in methyl alcohol solution, this methyl glucose gave a phenylhydrazone which had the same properties as the corresponding derivative of the 2-methyl glucose of Brigl and Schinle. Moreover, like their 2-methyl glucose, on heating with excess phenylhydrazine in dilute acetic acid solution, it lost the methyl group and gave glucosazone, and not a methyl hexosazone, as reported by Pacsu.

[1] Pacsu, E., *Ber. chem. Ges.*, **58**, 1455 (1925).

[2] Brigl, P., and Schinle, R., *Ber. chem. Ges.*, **63**, 2884 (1930).

Additional proof of the identity of the methyl glucose of Pacsu and the 2-methyl-*d*-glucose of Brigl and Schinle was afforded by the fact that their 1,1-diethylmercapto derivatives are similar.

These facts would suffice to establish the identity of Pacsu's supposed 4-methyl glucose with the 2-methyl glucose of Brigl and Schinle. However, for further confirmation, the structure of the substance was arrived at by demonstrating that the hydroxyls in positions 3, 4, 5, and 6 were unsubstituted. Position 3 had already been excluded by Pacsu, as his methyl glucose was different from the 3-methyl glucose prepared from diacetone glucose. Position 6 is excluded by us as the methyl glucose of Pacsu oxidizes to a methyl saccharic acid. Positions 4 and 5 are excluded as changes in the optical rotation of a methyl alcohol solution of the sugar, containing 0.5 per cent hydrogen chloride, indicate both furanoside and pyranoside formation. Thus, the methyl group must be attached to the hydroxyl of atom 2.

In agreement with this view is the fact that on oxidation by the method of Lehmann-Maquenne,[3] the sugar consumes only 0.87 equivalents of oxygen per mol. This value is close to those found by Sobotka[4] for substances substituted in position 2 (2,3-dimethyl glucose, 0.59; 2,3,4,6-tetramethyl glucose, 0.82) and quite different from those found when position 2 was unsubstituted (glucose, 5.16; 3-monomethyl glucose, 3.21; 3,5,6-trimethyl glucose, 1.98).

It may be questioned as to whether the monomethyl glucose of Pacsu is actually derived through a diacetone derivative, but if so then the latter must have position 2 open.

In addition, it may be mentioned that Pacsu claimed that the osazone of his methyl glucose was identical with that of methyl mannose prepared in a similar manner.[5] Granting this, it is warranted to conclude that in the methyl mannose of Pacsu, also, the methyl group is in position 2.

EXPERIMENTAL

Methyl Glucose (Pacsu)—1,1-dibenzylmercaptoglucose (m.p. 137°) was acetonated and then methylated according to the pro-

[3] See Griesbach, W., and Strassner, H., *Z. physiol. Chem.*, **88**, 199 (1913).

[4] Sobotka, H. H., *J. Biol. Chem.*, **69**, 267 (1926).

[5] Pacsu, E., and Kary, C., *Ber. chem. Ges.*, **62**, 2811 (1929).

cedure of Pacsu.[1] After removing the acetone residues by hydrolysis, the resulting methyl 1,1-dibenzylmercaptoglucose melted at 194°. The methyl glucose obtained after removal of the mercapto groups melted at 158°. Its rotation, 3 minutes after dissolving was $[\alpha]_D^{23} = +21.1°$ in water, and the rotation at equilibrium was

$$[\alpha]_D^{25} = \frac{+1.21° \times 100}{2 \times 0.97} = +62.1°$$

The analysis corresponded to that of a methyl hexose.

4.395 mg. substance: 6.955 mg. CO_2 and 2.900 mg. H_2O.
0.1010 gm. " : 0.1150 gm. AgI.

$C_7H_{14}O_6$.	Calculated.	C 43.27, H 7.27, OCH_3 15.98
	Found.	" 43.15, " 7.38, " 15.00

Pacsu reported a melting point of 156–157° and optical rotations of +18.57° after 3 minutes and +61.9° at equilibrium. Brigl and Schinle[2] gave the melting point as 158° and the rotations +56.6° after one-half hour and +65.6° at equilibrium.

The reducing value of the methyl glucose was determined by the Lehmann-Maquenne[3] method and in the same manner as the determinations of Sobotka[4] so as to be comparable with his results. A sample of 3-methyl glucose was run similarly to afford a check on the technique used.

29.1 mg. of the above methyl glucose required 1.3 cc. of 0.1 N thiosulfate or 0.87 equivalents of oxygen per mol and 29.1 mg. of 3-methyl glucose required 4.7 cc. or 3.14 equivalents per mol. Sobotka found 3.21 equivalents per mol for 3-methyl glucose.

Phenylhydrazone—To 1.0 gm. of the methyl glucose in a small crystallizing dish there was added a solution of 0.56 gm. of phenylhydrazine (1 mol per mol of sugar) in about 10 cc. of methyl alcohol, and the mixture was warmed on the steam bath and stirred. The sugar dissolved, giving an almost colorless solution. When nearly all the alcohol had been removed by evaporation, the dish was removed from the steam bath, and on further stirring the syrup crystallized as an almost solid mass. The product was once recrystallized from 5 per cent ethyl alcohol and was then per-

fectly colorless. The yield of recrystallized product was 0.25 gm. The analysis corresponded to a phenylhydrazone of a methylhexose.

4.020 mg. substance: 0.344 cc. N (757 mm. and 25°).
4.655 " " : 4.175 mg. AgI.

$C_{13}H_{20}O_5N_2$.	Calculated.	N 9.86,	OCH_3 10.92
	Found.	" 9.76,	" 11.79

The melting point was 177–178° with no decomposition. The rotation, 3 minutes after dissolving was

$$[\alpha]_D^{25} = \frac{-0.36° \times 100}{2 \times 2.0} = -9.0° \text{ (in pyridine)}$$

The solution, which was originally colorless became rapidly yellow so that the end value (+8.0°) is probably not significant. Brigl and Schinle[2,6] report 177° and 178° as the melting points of the hydrazone of 2-methyl glucose and a specific rotation in pyridine of −12.3 to −13.3°. Hickinbottom[7] gave the melting point as 175–176° and Lieser[8] 176° (uncorrected).

Phenylosazone—To 2 gm. of the methyl glucose in about 100 cc. of water were added 3.4 gm. of phenylhydrazine (3 mols per mol of sugar) and 3.5 cc. of glacial acetic acid. The mixture was heated on the steam bath and the osazone soon began to crystallize. Its rate of formation was slower, however, than from an equivalent weight of glucose and although the mixture was heated for 1½ hours, the yield was much less than would have been expected from the same amount of glucose. The solution was cooled, the osazone filtered off and washed with methyl alcohol. It was then a felt of light canary-yellow needles. Yield 0.3 gm. The analysis corresponded to that of a hexosazone, a micro methoxy determination being entirely negative after heating for 2 hours.

4.106 mg. substance: 0.572 cc. N (757 mm. and 26°).
$C_{18}H_{22}O_4N_4$. Calculated. N 15.64. Found. N 15.83

The melting point was 208–210° as was that of an intimate mixture with a sample of pure glucosazone. The rotation was

[6] Brigl, P., and Schinle, R., *Ber. chem. Ges.*, 62, 1716 (1929).
[7] Hickinbottom, W. J., *J. Chem. Soc.*, 3140 (1928).
[8] Lieser, T., *Ann. Chem.*, 470, 104 (1929).

$-78°$ after 15 minutes and $[\alpha]_D^{23} = \frac{-0.05° \times 100}{0.5 \times 0.33} = -30°$ at equilibrium (in pyridine-absolute alcohol, 2:3 by volume).

Pacsu described his osazone as melting at 198° and as having an initial rotation of $-50.3°$ and a final one of $-34.8°$. The analysis of his product corresponded quite well to that of a methyl hexosazone, but he made no methoxyl determination and the calculated analysis of a methyl hexosazone is not very different from that of the simple hexosazone.

$C_{18}H_{22}O_4N_4$.	Calculated.	C 60.31, H 6.19, N 15.64
$C_{19}H_{24}O_4N_4$.	"	" 61.25, " 6.48, " 15.06
	Found (Pacsu).	" 61.30, " 6.41, " 15.23

Methyl-1,1-Diethylmercaptoglucose—The procedures given by Pacsu were employed to obtain the methyl glucose from diethylmercaptoglucose instead of from dibenzylmercaptoglucose. The methyl-1,1-diethylmercaptoglucose obtained in this way melted at 157–158° and had a rotation of

$$[\alpha]_D^{25} = \frac{-1.51° \times 100}{2 \times 3.0} = -25.2° \text{ (in pyridine)}$$

The analysis was as follows:

4.810 mg. substance: 7.750 mg. CO_2 and 3.410 mg. H_2O.
3.655 " " : 5.667 " $BaSO_4$.

$C_{11}H_{24}O_5S_2$.	Calculated.	C 43.97, H 8.06, S 21.32
	Found.	" 43.93, " 7.93, " 21.30

Brigl and Schinle reported a rotation of $[\alpha]_D^{20} = -25.0°$ and a melting point[9] of 178°. Papadakis[10] gave melting points of 156–157° and 155°.

The same mercapto compound resulted on treating Pacsu's methyl sugar (prepared through the dibenzylmercaptoglucose) with ethyl mercaptan. 3.5 gm. of the methyl glucose were shaken with 3.5 cc. of concentrated hydrochloric acid (*d* 1.12) and 2.0

[9] As Brigl and Schinle state that their product is identical with that of Papadakis, and that mixed melting points show no depression, this value is perhaps due to a typographical error.

[10] Papadakis, P. E., *J. Am. Chem. Soc.*, **52**, 2147, 3465 (1930).

cc. of freshly distilled ethyl mercaptan until the mixture solidified, about 20 minutes. The solid was broken up and stirred with water, then filtered, and washed with water. The solid was recrystallized several times from 95 per cent alcohol. The product analyzed for a methyl diethylmercaptohexose.

4.959 mg. substance: 8.010 mg. CO_2 and 3.475 mg. H_2O.
5.144 " " : 7.973 " $BaSO_4$.
$C_{11}H_{24}O_5S_2$. Calculated. C 43.97, H 8.06, S 21.32
Found. " 44.04, " 7.84, " 21.29

The substance melted at 157° and its rotation was

$$[\alpha]_D^{25} = \frac{-1.01° \times 100}{2 \times 1.98} = -25.5° \text{ (in pyridine)}$$

Glucoside Formation—The glucoside formation in methyl alcohol containing 0.5 per cent of hydrogen chloride and 0.34 mols per

TABLE I

Observed Rotations during Glucoside Formation at 25°

Time		Rotation	Time		Rotation	Time	Rotation
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*degrees*
0	12	0.89	4	00	0.62	96	−0.12
0	30	0.84	7	30	0.36	172	−0.05
1	00	0.75	24	00	0.10	264	0.00
2	00	0.68	48	00	−0.06		

liter of the sugar was observed by following the changes in rotation of the solution. Experiments were made at room temperature (20–25°) and in baths of boiling chloroform and carbon tetrachloride. In the last two cases samples were sealed in glass tubes, cooled in ice, and after being in the bath for the assigned time, were again ice-cooled until the reading was to be made. They were then quickly warmed to room temperature and read. In all cases the readings were made at 23–25° in a 2 dm. tube with sodium D light. The readings are given in Tables I, II, and III and the specific rotations calculated from them are plotted in Fig. 1.

Methyl Saccharic Acid.—2 gm. of the methyl glucose were

TABLE II

Observed Rotations during Glucoside Formation at 61°

Time		Rotation	Time		Rotation	Time		Rotation
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*min.*	*degrees*
0	00	0.99	1	15	−0.08	17	30	0.70
0	10	0.14	2	40	+0.08	27	00	0.92
0	20	0.03	5	00	0.25	38	00	1.01
0	40	−0.15	9	16	0.48	49	45	1.07

TABLE III

Observed Rotations during Glucoside Formation at 75°

Time		Rotation	Time		Rotation	Time		Rotation
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*min.*	*degrees*
0	00	0.99	0	50	0.15	10	00	0.95
0	05	0.07	1	30	0.34	19	30	1.16
0	10	−0.09	3	00	0.57	33	00	1.22
0	20	+0.01	5	00	0.74	49	45	1.23

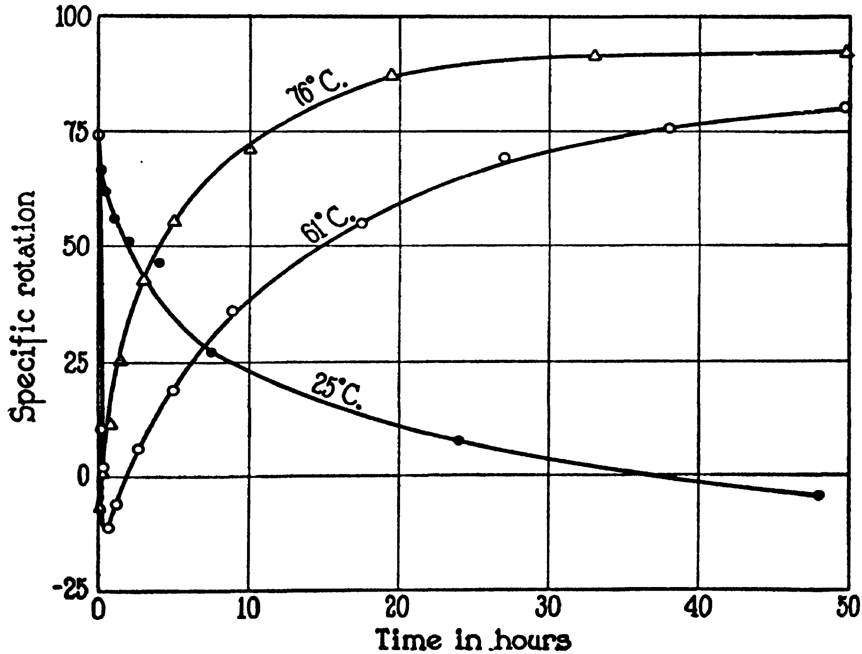

FIG. 1. Specific rotations during glucoside formation

oxidized with nitric acid as described by Levene and Meyer[11] for 3-methyl glucose.

After evaporating off all the nitric acid the residue was twice evaporated with water, then dissolved and converted to the calcium salt. This was purified by repeated precipitation from water with alcohol.

The analysis corresponded to the calcium salt of a methyl saccharic acid.

10.125 mg. substance: 5.215 mg. $CaSO_4$.
9.820 " " : 8.660 " AgI.

$C_7H_{10}O_8Ca$.	Calculated.	Ca 15.28,	OCH_3 11.83
	Found.	" 14.99,	" 11.64

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[11] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **54**, 805 (1922).

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Vol. 91, No. 2, pp. 751–760]

HEXOSEMONOPHOSPHATE (ROBISON)

NATURAL AND SYNTHETIC

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(Received for publication, March 27, 1931)

In a previous communication[1] we described a synthetic glucosephosphate, prepared through monoacetone glucose, and concluded that its structure was glucose-6-phosphate as its osazone was identical with that of the Harden-Young and Neuberg esters. At the same time we pointed out that in other properties, including enzymic fermentation, it was quite like the Robison ester. The assignment of the structure of glucose-6-phosphate to the Robison ester was supported by all the available data with the single exception of the osazone, that of the Robison ester having been described[2] as melting at 139° while that of the Harden-Young and Neuberg[3] esters melted at 151–152°. We pointed out that to us the only apparent alternatives were either that the osazone described by Robison was impure and consequently had a low melting point, or else that the two glucosides which can be formed from the Robison ester are not 1, 4 and 1, 5 as is the case of ordinary sugars.

In order to establish conclusively the fact that positions 4 and 5 are unoccupied in the Robison ester, we have undertaken a study of the lactone formation of the aldonic acid derived from it. At the same time we prepared the aldonic acids from our synthetic glucose-6-phosphate and from the synthetic glucose-3-phosphate. All three acids exhibit the formation of two lactones so that posi-

[1] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **89**, 479 (1930).

[2] Robison, R., *Biochem. J.*, **16**, 809 (1922).

[3] von Lebedev, A., *Biochem. Z.*, **28**, 213 (1910). Young, W. J., *Biochem. Z.*, **32**, 177 (1911).

tions 4 and 5 are not occupied in any of them (this was previously shown[1] for the glucose-3-phosphate), substantiating the results with the glucosides. Moreover, the Robison ester behaves in all respects like the synthetic 6-phosphate and is quite different from the 3-phosphate, confirming the assignment of the structure of glucose-6-phosphate to the Robison ester.

This leaves unanswered only the matter of the melting points of the osazone. As previously mentioned, we were able to obtain only the osazone of the Harden-Young ester when we used an impure preparation of the Robison ester. The experiment has just now been repeated by Robison and King[4] on a truly pure preparation of the Robison ester and they likewise find that the osazone is identical with that from the Harden-Young ester and melts at 154–154.5° instead of 139° as originally reported by Robison.

Thus all results are in harmony with the assignment of the structure of glucose-6-phosphate to the Robison ester.

The synthesis of the Robison ester reported in the earlier paper may be useful when this ester is required for biological studies; a possibly easier synthesis will be described later.

EXPERIMENTAL

A. Phosphate from Monoacetone Glucose—Recrystallized monoacetone glucose was phosphorylated as previously described[1] and the acetone group was hydrolyzed off as before with hydrochloric acid. The chloride was removed with freshly prepared silver sulfate, the residual silver was precipitated with hydrogen sulfide, and the excess hydrogen sulfide was removed by aeration. A warm saturated barium hydroxide solution was added until the mixture was just alkaline to phenolphthalein, and it was then centrifuged. The solution was concentrated under reduced pressure to about half volume, filtered with charcoal, and then further concentrated. The barium salt was precipitated with an equal volume of 95 per cent alcohol, washed with 50 per cent alcohol, redissolved, and reprecipitated in the usual way. The dried salt was dissolved in water and the barium was quantitatively removed with sulfuric acid. After centrifuging off the barium sulfate, a methyl alcohol solution of brucine was added to pH 7.0 to 7.2.

[4] Robison, R., and King, E. J., *J. Soc. Chem. Ind., Chem. and Ind.*, 50, 156 (1931).

After filtering the mixture with charcoal and concentrating to a small volume, 3 volumes of acetone were added. On scratching and standing, the product crystallized. It was filtered off, washed with 75 per cent acetone and then acetone, and was air-dried. It was recrystallized, but with difficulty, by dissolving in hot 90 per cent methyl alcohol and then adding 3 volumes of acetone.

Material crystallized three times had the following analysis.

6.340 mg. substance: 0.304 cc. N (755 mm. and 26.5°).
10.450 " " : 22.555 mg. ammonium phosphomolybdate (Pregl).

$C_{52}H_{65}O_{17}N_4P$.	Calculated.	N 5.34,	P 2.96
	Found.	" 5.42,	" 3.13

and its rotation was

$$[\alpha]_D^{25} = \frac{-0.83° \times 100}{1 \times 5.0} = -16.6° \text{ (in water)}$$

$$[\alpha]_D^{25} = \frac{-0.78° \times 100}{1 \times 5.0} = -15.6° \text{ (in pyridine-water, 1:1 by volume)}$$

Some of this material was dissolved in water, and a barium hydroxide solution was added until the mixture was just alkaline to phenolphthalein. The brucine was filtered off and washed with water, and to the combined filtrate and washings a little more barium hydroxide solution was added to restore the pH to about 8.5. The solution was concentrated to a small volume, filtered with charcoal, and precipitated with an equal volume of 95 per cent alcohol. The subsequent washing, solution, reprecipitation, and drying were done in the usual manner.

The analysis corresponded to a barium hexosemonophosphate.

0.0948 gm. substance: 0.0536 gm. $BaSO_4$.
4.110 mg. " : 23.760 mg. ammonium phosphomolybdate (Pregl).

$C_6H_{11}O_9PBa$.	Calculated.	Ba 34.74,	P 7.84
	Found.	" 33.27,	" 8.39

and its rotation was

$$[\alpha]_D^{25} = \frac{+1.57° \times 100}{2 \times 4.74} = +16.6° \text{ (in water)}$$

From 2.0 gm. of this material the barium was quantitatively removed with sulfuric acid and the osazone was prepared as previously described. The analysis of the purified osazone was as follows:

4.000 mg. substance: 0.533 cc. N (753 mm. and 26°).
3.635 mg. " : 13.930 mg. ammonium phosphomolybdate (Pregl).

$C_{24}H_{31}O_7N_6P$. Calculated. N 15.38, P 5.68
Found. " 15.07, " 5.56

It melted at 151–152° as did a mixture with the osazone from the Harden-Young diphosphate. Its rotation, in pyridine-absolute alcohol (2:3 by volume), was −43.3° after 15 minutes and

$$[\alpha]_D^{25} = \frac{-0.17° \times 100}{0.5 \times 1.2} = -28.3° \text{ (at equilibrium)}$$

A portion of the barium salt was oxidized to the aldonic acid with barium hypoiodate. 12 gm. of the dry barium salt were dissolved in 60 cc. of water and added to a solution of 7.8 gm. of iodine and 15 gm. of barium iodide in 30 cc. of water. The mixture was stirred with a mechanical stirrer and 240 cc. of a 0.5 N barium hydroxide solution were run in through a dropping funnel over a period of 20 minutes. The mixture was stirred for an additional 15 minutes, made acid with sulfuric acid, and sulfur dioxide was passed in until the iodine was all reduced. Barium hydroxide was added to pH about 8.5 and the precipitate was centrifuged off and washed with warm water until the washes gave a negative test for halides. The precipitate was then suspended in water, stirred mechanically, and sulfuric acid was added in small portions until the barium was all removed. Barium hydroxide solution was cautiously added until the solution contained neither sulfate nor barium, and the mixture was then centrifuged. To the solution a methyl alcohol solution of brucine was added to pH 7.0 to 7.2 and the mixture was filtered with charcoal. It was concentrated under reduced pressure to a volume of about 125 cc. and ½ volume of methyl alcohol was added. On standing overnight at 10–15°, the material crystallized in beautiful needles. Further crops were obtained by cooling the mixture and finally by concentrating and cooling. The combined material was twice recrystallized from 50 per cent (by volume) methyl alcohol.

The analysis corresponded to a tribrucine salt of a phosphoaldonic acid.

6.689 mg. substance: 0.328 cc. N (756 mm. and 25°).
4.963 " " : 7.921 mg. ammonium phosphomolybdate (Pregl).
$C_{75}H_{90}O_{22}N_6P$. Calculated. N 5.77, P 2.13
Found. " 5.59, " 2.36

and its rotation was

$$[\alpha]_D^{25} = \frac{-1.16° \times 100}{2 \times 1.94} = -29.9° \text{ (in water; dissolved by warming and then cooled)}$$

$$[\alpha]_D^{25} = \frac{-2.58° \times 100}{2 \times 4.84} = -26.7° \text{ (in pyridine-water, 1:1 by volume)}$$

The product was dissolved in warm water and cooled, and sodium hydroxide solution was added, very cautiously, to pH 8.0 to 8.2. The brucine was filtered off and washed with water, and the pH of the combined filtrate and washings was readjusted to 8.0 with more sodium hydroxide. The solution was concentrated under reduced pressure to a small volume, filtered with charcoal, and further concentrated. The material was placed in a desiccator until it became syrupy and was then ground several times with absolute alcohol. It was dried over calcium chloride and then to constant weight over phosphorus pentoxide under reduced pressure. The final product was a granular, colorless substance, the phosphorus content of which corresponded to the trisodium salt of a phosphoaldonic acid.

3.890 mg. substance: 24.900 mg. ammonium phosphomolybdate (Pregl).
$C_6H_{10}O_{10}PNa_3$. Calculated. P 9.07
Found. " 9.29

Its rotation in water was

$$[\alpha]_D^{25} = \frac{+0.19° \times 100}{2 \times 5.0} = +1.9°$$

0.25 gm. of this material was dissolved in water, 1.68 cc. of 1.0 N hydrochloric acid were added (2.3 equivalents per mol), the mixture was diluted to a volume of 5.0 cc., and its rotation was

measured in a 2 dm. tube with sodium (D) light. The readings (at a temperature of 25°) are given in Table I.

The specific rotations were calculated from these data and are

TABLE I

Observed Rotations during Lactone Formation

Glucose-3-phosphate			Glucose-6-phosphate			Robison monophosphate		
Time		Rotation	Time		Rotation	Time		Rotation
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*min.*	*degrees*
0	1½	−1.63	0	1½	−0.29	0	1½	−0.30
0	3	−1.56	0	3	−0.20	0	3½	−0.17
0	7	−1.47	0	6	−0.11	0	6	−0.13
0	15	−1.38	0	10	−0.06	0	11	−0.07
0	31	−1.24	0	17	−0.06	0	26	−0.03
1	8	−1.15	0	37	−0.04	1	6	+0.02
2	7	−1.04	1	17	+0.01	4	0	+0.14
4	30	−0.98	3	45	+0.10			

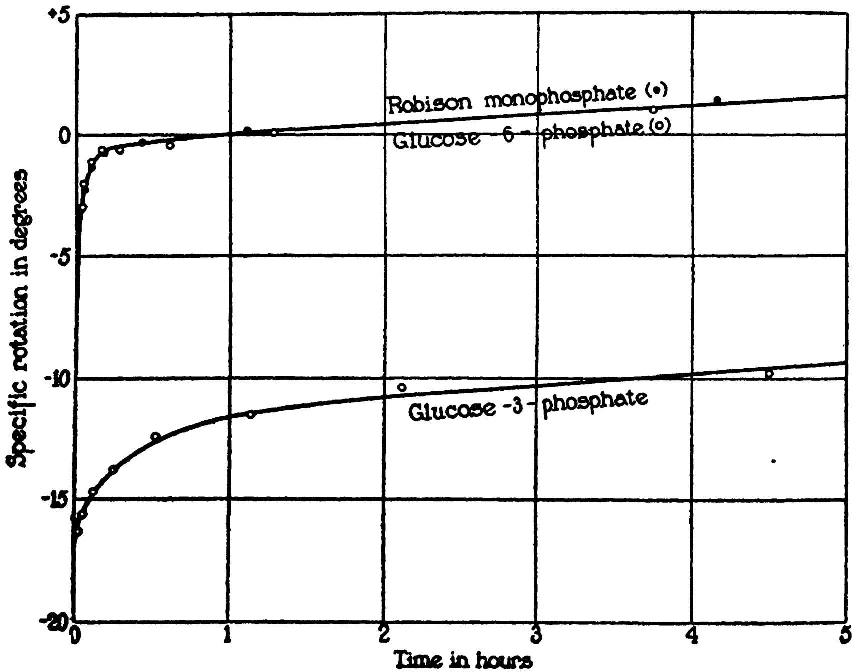

FIG. 1. Observed rotations during lactone formation

given in Fig. 1. The specific rotations are calculated in terms of sodium salt, and not of acid, in order to avoid any question as to the completeness of the reaction between the hydrochloric acid and the salt.

B. Phosphate from Diacetone Glucose—The preparation of this ester has been previously described, and the properties of the barium and brucine salts have been given. The only additional information which need be given here is the rotation of the brucine salt in pyridine-water which is

$$[\alpha]_D^{25} = \frac{-1.45° \times 100}{2 \times 5.0} = -14.5° \text{ (in pyridine-water, 1:1 by volume)}$$

The osazone, previously described,[5] is an anhydrohexosazone free of phosphorus, the phosphate group having been eliminated during the osazone formation.

The oxidation to the acid with barium hypoiodate, and the conversion to the brucine salt were carried out exactly as described above. The brucine salt behaved a little differently, however, and was twice crystallized from 90 per cent (by volume) methyl alcohol. Its analysis corresponded to a dibrucine salt of a phosphoaldonic acid.

5.400 mg. substance: 0.260 cc. N (747 mm. and 25°).
3.810 " " : 8.050 mg. ammonium phosphomolybdate (Pregl).
$C_{52}H_{65}O_{18}N_4P$. Calculated. N 5.26, P 2.98
Found. " 5.43, " 3.07

Addition of more brucine and recrystallization of the product again led to only a dibrucine salt.

The rotation was

$$[\alpha]_D^{25} = \frac{-0.98° \times 100}{2 \times 2.0} = -24.5° \text{ (in water; dissolved by warming and then cooled)}$$

$$[\alpha]_D^{25} = \frac{-1.55° \times 100}{2 \times 5.0} = -15.5° \text{ (in pyridine-water, 1:1 by volume)}$$

The brucine salt was converted to the sodium salt. Its phosphorus content corresponded to that of a trisodium salt of a phosphoaldonic acid.

[5] Levene, P. A., Raymond, A. L., and Walti, A., *J. Biol. Chem.*, **82**, 191 (1929).

3.885 mg. substance: 23.600 mg. ammonium phosphomolybdate (Pregl).
$C_6H_{10}O_{10}PNa_2$. Calculated. P 9.07
Found. " 8.81

Its rotation was

$$[\alpha]_D^{25} = \frac{+0.13^\circ \times 100}{2 \times 5.0} = +1.3^\circ \text{ (in water)}$$

Hydrochloric acid to the extent of 2.3 equivalents per mol was added to a solution of the sodium salt as in the case of the previous ester. The concentrations were the same and the rotations, measured at 25° in a 2 dm. tube with sodium (D) light, are given in Table I. The specific rotations calculated from these are included in Fig. 1 for comparison. They are almost identical with those previously recorded,[1] the difference being due to the greater acidity used in the present experiments.

C. Robison Monophosphate—This ester was prepared by fermenting a mixture of 4 parts of glucose and 1 part of fructose with yeast juice, phosphate being added every few minutes to maintain a maximum fermentation rate. The phosphate was followed colorimetrically and was kept between 0.01 and 0.04 M. Considerable difficulty was experienced with the procedure as the fermentation and phosphorylation varied greatly from batch to batch and for no assignable reason. Variations of temperature and concentrations gave no consistent results, and finally a large number of preparations were made in an entirely arbitrary fashion. In working up the material, the protein was precipitated with 6 per cent of trichloroacetic acid, and the hexosediphosphate and unchanged phosphate were precipitated from the filtrate by adding barium acetate and then barium hydroxide until alkaline to phenolphthalein. The mixture was filtered, the precipitate washed with water, and the filtrate and washings were combined. Basic lead acetate was added to complete precipitation, the pH being adjusted to about 8 after each addition. The lead salt was well washed with water, suspended in water, and decomposed with hydrogen sulfide. The excess of the gas was removed by thorough aeration, barium hydroxide solution was added to pH about 8.5, and the solution was filtered with charcoal. It was then concentrated under reduced pressure to a small volume and refiltered

with charcoal. The barium salt was precipitated by the addition of an equal volume of 95 per cent alcohol and was washed with 50 per cent and then with 95 per cent alcohol. It was twice suspended in acetone and filtered and was then dried in air at about 40°. The product as thus prepared, contained, according to a Willstätter hypoiodate titration, about 50 per cent of hexosemonophosphate.

It was oxidized with barium hypoiodate exactly as in the case of the synthetic esters except that the amount of ester was changed to correspond to its reduced aldose content, approximately twice as much ester being used for the same quantities of the other reagents. The isolation of the barium salt and the conversion to the brucine salt were carried out exactly as in the other cases. The properties of the brucine salt were similar to those of the product from the monoacetone and the recrystallizations were performed similarly.

The analysis of the product corresponded to that of a tribrucine salt of a phosphoaldonic acid.

6.780 mg. substance: 0.344 cc. N (764 mm. and 24°).
5.890 " " : 8.920 mg. ammonium phosphomolybdate (Pregl).

$C_{75}H_{90}O_{22}N_6P$. Calculated. N 5.77, P 2.13
Found. " 5.86, " 2.21

The rotation was

$$[\alpha]_D^{25} = \frac{-1.22° \times 100}{2 \times 1.975} = -30.9° \text{ (in water; dissolved by warming and then cooled)}$$

$$[\alpha]_D^{25} = \frac{-2.74° \times 100}{2 \times 4.94} = -27.8° \text{ (in pyridine-water, 1:1 by volume)}$$

This material was converted to the sodium salt as before, the analysis of this substance corresponding to that of a trisodium salt of a phosphoaldonic acid.

4.707 mg. substance: 28.820 mg. ammonium phosphomolybdate (Pregl).
$C_6H_{10}O_{10}PNa_3$. Calculated. P 9.07. Found. P 8.89.

The rotation in water was

$$[\alpha]_D^{25} = \frac{+0.06° \times 100}{2 \times 5.0} = +0.6°$$

Hydrochloric acid to the extent of 2.3 equivalents per mol was added to a solution of the sodium salt as in the case of the previous esters. The concentrations were the same, and the rotations, measured at 25° in a 2 dm. tube with sodium (D) light, are given in Table I. The specific rotations calculated from these data are included in Fig. 1.

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ON WALDEN INVERSION

XVI. THE INFLUENCE OF SUBSTITUTING GROUPS ON OPTICAL ROTATION IN THE SERIES OF DISUBSTITUTED PROPIONIC ACIDS CONTAINING AN ETHYL GROUP

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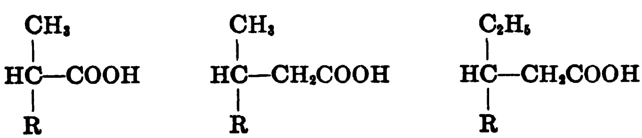
The primary object of the present investigation was to obtain further data on the effect on the optical rotation of the substitution of a hydroxyl by a halogen. Incidentally, it was hoped to obtain additional data which might lead to a deeper understanding of the relationship between chemical structure and optical activity.

Three series of substances have now been studied in which the substitution is accomplished definitely without Walden inversion inasmuch as in these substances the group to be substituted is allocated not directly on the asymmetric carbon atom. The differences in the structures of the substances in the three series can be seen from the following figures, which represent the three parent substances



I II III

(Group R being aliphatic and higher than methyl in Series I and II and higher than ethyl in Series III).

Each member of Series I differs from that of Series II by one link, CH_2, between the polar group and the asymmetric carbon atom. Series II and III differ by the character of one of the groups attached to the asymmetric carbon atom. This group is a

methyl in Series II and an ethyl in Series III. Thus, a comparison of the three series permits the study, on one hand, of the effect on the rotation of a given polar group as a function of its distance from the asymmetric carbon atom, and on the other hand, of the respective effects on the rotation of the groups

$$\begin{array}{l} CH_3 \\ | \\ HC- \\ | \\ R \end{array} \quad \text{and} \quad \begin{array}{l} C_2H_5 \\ | \\ HC- \\ | \\ R \end{array}$$

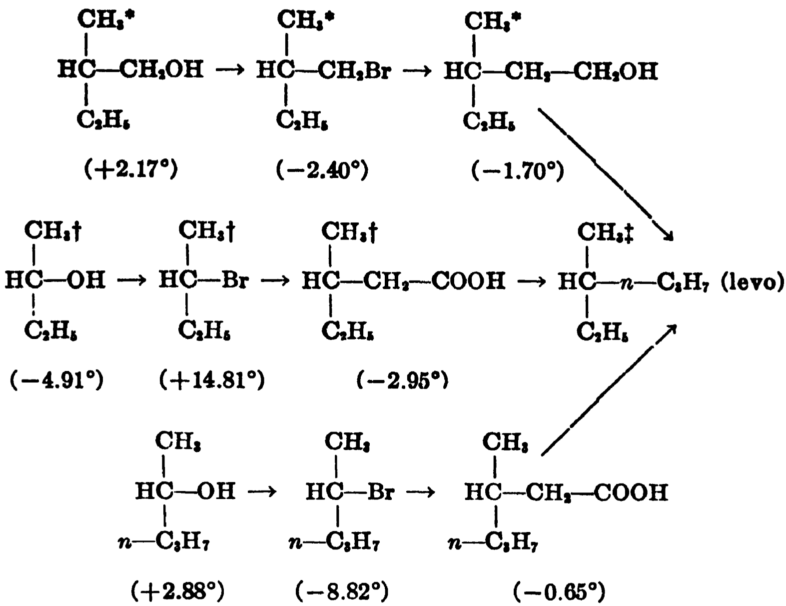
Configurational Relationship of the Substances of the Three Series—Before the effect of substitution on the rotation can be studied intelligently, it is necessary first to correlate the configurations of the members of each series among themselves and second, to correlate the configurations of the series among each other.

Series I—In the series of aliphatic disubstituted acetic acids all members on substitution of one polar group by another behave similarly. The parent-substituted acetic acids rotate in the same direction and, therefore, there was no reason to suspect any configurational differences between the individual disubstituted acetic acids (the substituting groups being aliphatic) rotating in the same direction.

Series II—In this series the first member, namely 1,1-methylethylpropionic acid (3), and the higher members were configurationally related when they rotated in opposite directions. Evidence to this effect was produced in a previous paper by Levene and Marker.[1] Additional evidence is furnished in this communication; namely, it is now shown that dextro-methylethyl carbinol leads to dextro-1,1-methylethylpropionic acid (3) whereas the configurationally related dextro-methylpropyl carbinol leads to the levo-1,1-methylpropylpropionic acid (3) (see the accompanying set of figures).

Series III—Considering 1,1-ethylpropylpropionic acid (3) as the first member of the series, it was found that the levorotatory first member of the series was configurationally related to the dextrorotatory higher members. The evidence for this view is anal-

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 91, 77 (1931).

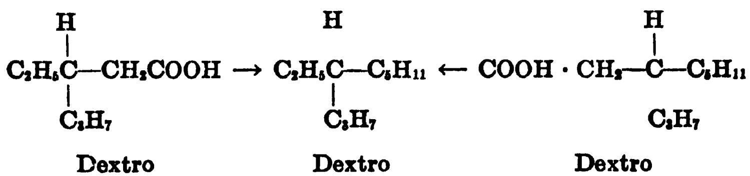


* For convenience of discussion the signs of rotation of all members of this series were changed from those found experimentally. All the values are in $[M]_D^{23 \pm 2°}$ (in degrees).

† Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 91, 405 (1931).

‡ For intermediate steps from acids or carbinols to hydrocarbons see Levene and Marker.[1]

ogous to that which establishes the configurational relationship of the first and of the higher members of Series II. Namely, it was shown that dextro-1,1-ethylpropylpropionic acid (3) and dextro-1,1-ethylamylpropionic acid (3) lead to the same dextrorotatory ethylpropylamyl methane, as can be seen from the following figures.



The second method was not quite so successful in this series for the reason that the reaction on condensation of dextro-ethylpropyl

TABLE I

M_D^{25} (*in Degrees*)

	—OH	—Br	—CH_2COOH	—$CH_2COOC_2H_5$	—CH_2CH_2OH	—CH_2CH_2Br	—CH_2CH_2Cl	—$CH_2CH_2CHOHC_2H_5$	—n—C_5H_{11}	—$CH_2CH_2CH_2OH$	—n—C_3H_7
CH_3—HC—C_2H_5	+4.91	−14.81	+2.95								
			+4.21	+4.69	+3.69		+8.95				
C_2H_5—HC—n—C_3H_7			+2.67	+1.02	+0.64	+1.97	+1.68	+0.56	+1.09		
	+1.70	−3.35	Racemized								
C_2H_5*—HC—n—C_4H_9	−8.56	+16.38	+2.13	+1.04	+0.95	+0.66					
C_2H_5*—HC—n—C_5H_{11}	−8.87	+21.32	+2.87	+1.53	+1.82	+1.35					
						+1.52				0	+0.76

* For convenience of discussion, the signs of rotation of all members of this series were changed from those found experimentally.

carbinol with malonic ester was accompanied by racemization. On the other hand, the dextro-ethylbutyl and dextro-ethylamyl-bromomethanes led to levo-1,1-disubstituted propionic acid (3), resembling in this respect the behavior of the corresponding members of Series II (see Table I).

Configurational relationships between the series of disubstituted acetic and 1,1-disubstituted propionic acids (3) have been established by the fact that the dextrorotatory methylethylpropylmethane obtained by Marckwald[2] by condensation of dextro-2-ethylpropyl iodide (3) with ethyl iodide was identical with the one obtained by us from the dextrorotatory 2-ethylbutyl bromide (4). In this manner it was shown that the series of substances given in the first column is related to the series given in the second column.

The configurational relationships of the substances given in the second and the third columns may be seen from the fact that dextro-methylpropyl carbinols lead to levo-1,1-methylpropyl-propionic acid (3) and the configurationally related dextro-ethylbutyl and ethylamyl carbinols lead to levorotatory disubstituted propionic acids.

Thus, the substances given in Table II are configurationally related and therefore the group of substances may serve in the analysis of the factors determining the direction and the relative values of the rotations of simple substances.

Effect of Polarities of Substituting Groups on Optical Activity—Upon comparison not only of the directions of the rotation but also the values of individual rotations, as given in the following figures, it is found that in Series I

$$\begin{array}{ccc} \left.\begin{array}{l} CH_3 \\ C_2H_5 \end{array}\right\rangle CHCOOH & \left.\begin{array}{l} CH_3 \\ C_2H_5 \end{array}\right\rangle CHCH_2Br & \left.\begin{array}{l} CH_3 \\ C_2H_5 \end{array}\right\rangle CHCH_2OH \\ -19.03^\circ & -7.9^\circ & +5.19^\circ \end{array}$$

the acid has the highest levorotation, the bromide has a lower levorotation, and the carbinol is dextrorotatory. Thus with respect to this influence on rotation, the substituting groups can be arranged in the following order $COOH < CH_2Br < CH_2OH$ according to the increasing dextrorotation. It has been pointed

[2] Marckwald, W., *Ber. chem. Ges.*, **37**, 1046 (1904).

out in previous communications from this laboratory[3] and earlier by Rule[4] that this order of arrangement is analogous to that of their directive effect on the substitution in the benzene nucleus, or in order of their specific inductive capacities.

In the derivatives of the 1,1-disubstituted propionic acids (3), the acids possess the highest dextrorotation and the bromides are levorotatory (as in Series II) or possess the lowest dextrorotation (as in Series III) as can be seen from the following figures.

$$\overset{CH_3}{\underset{C_4H_9}{>}}CHCH_2COOH \rightarrow \overset{CH_3}{\underset{C_4H_9}{>}}CHCH_2CH_2OH \rightarrow \overset{CH_3}{\underset{C_4H_9}{>}}CHCH_2CH_2Br$$

$$+5.49^\circ \qquad\qquad +3.58^\circ \qquad\qquad +15.19^\circ$$

$$\overset{C_2H_5}{\underset{C_4H_9}{>}}CHCH_2COOH \rightarrow \overset{C_2H_5}{\underset{C_4H_9}{>}}CHCH_2CH_2OH \rightarrow \overset{C_2H_5}{\underset{C_4H_9}{>}}CHCH_2CH_2Br$$

$$+2.13^\circ \qquad\qquad +0.95^\circ \qquad\qquad +0.66^\circ$$

Thus, arranging the substituting group in a manner analogous to that of the arrangement of Series I, the following order is obtained. $CH_2Br < CH_2OH < COOH$. Thus in this series the acid has the highest dextrorotation whereas in the previous series the acid possessed the highest levorotation. The order $Br > OH > COOH$ is the order of the effect of the groups on the dissociation constants of substituted acids.

The significant feature of these observations, however, is the fact that whenever the polar groups are located near the asymmetric carbon atom, the halides rotate in opposite direction from the configurationally related carbinols (as in Series I and II) or at least show a drop in the numerical value of the rotation (as in Series III) which also means a change towards the opposite direction.

If the same rule were applied for the correlation of the configurations of secondary carbinols with the corresponding halides, for instance,

[3] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84**, 571 (1929).
[4] Rule, H. G., *J. Chem. Soc.*, **125**, 1121 (1924).

TABLE II

	CH_3—HC—CH_3 CH_2CH_2Br 0 CH_2CH_2OH 0 CH_2COOH 0	C_2H_5—HC—CH_3 CH_2CH_2Br +9.2 CH_2CH_2OH +3.7 CH_2COOH +4.1	C_3H_7—HC—CH_3 CH_2CH_2Br +16.8 CH_2CH_2OH − 1.4 CH_2COOH − 2.8
CH_3—HC—C_2H_5 CH_2Br − 7.9 CH_2OH + 5.2 COOH −19.0	CH_3—HC—C_2H_5 CH_2CH_2Br −9.2 CH_2CH_2OH −3.7 CH_2COOH −4.1	C_2H_5—HC—C_2H_5 CH_2CH_2Br 0 CH_2CH_2OH 0 CH_2COOH 0	C_3H_7—HC—C_2H_5 CH_2CH_2Br +2.0 CH_2CHOH +0.7 CH_2COOH +2.7
CH_3—HC—C_3H_7 CH_2Br −1.4 CH_2OH +1.7 COOH −6.8	CH_3—HC—C_3H_7 CH_2CH_2Br −16.8 CH_2CH_2OH + 1.4 CH_2COOH + 2.8	C_2H_5—HC—C_3H_7 CH_2CH_2Br −2.0 CH_2CH_2OH −0.7 CH_2COOH −2.7	C_3H_7—HC—C_3H_7 CH_2CH_2Br 0 CH_2CH_2OH 0 CH_2COOH 0
CH_3—HC—C_4H_9 CH_2Br *l* CH_2OH +1.1 COOH −6.3	CH_3—HC—C_4H_9 CH_2CH_2Br −15.2 CH_2CH_2OH + 3.6 CH_2COOH + 5.5	C_2H_5—HC—C_4H_9 CH_2CH_2Br +0.7 CH_2CH_2OH +1.0 CH_2COOH +2.1	C_3H_7—HC—C_4H_9 CH_2CH_2Br *l* CH_2CH_2OH " CH_2COOH "
CH_3—HC—C_7H_{15} CH_2Br − 2.93 CH_2OH + 5.95 COOH −14.18	CH_3—HC—C_5H_{11} CH_2CH_2Br −13.0 CH_2CH_2OH + 5.4 CH_2COOH + 7.1	C_2H_5—HC—C_5H_{11} CH_2CH_2Br +1.5 CH_2CH_2OH +1.8 CH_2COOH +2.9	C_3H_7—HC—C_5H_{11} CH_2CH_2Br *d* CH_2CH_2OH " CH_2COOH "
CH_3—HC—$C_{10}H_{21}$ CH_2Br − 0.97 CH_2OH + 3.43 COOH −12.67			

methylethyl carbinol and methylethylbromomethane, then the conclusion should be reached that the dextro-carbinol is correlated to the levo-halide. The value of the rotation of the halide, 31.98°, being higher than that of the carbinol which is 13.87°, it follows therefore that the halide rotating to the left of the dextro-carbinol can be the levorotatory only. The same conclusion was reached by Levene and Mikeska[3] *on other grounds.*

Exceptional Position of the Derivatives of the 1,1-Methylethyl- and of the 1,1-Ethylpropylacetic Acids—Upon comparison of the rotations of the 1,1-methylethylacetic acid and of its derivatives (see Table II) it is seen that they differ from the rotations of the other members of the same series in two respects. First, all three substances rotate in one direction, whereas in the derivatives of the other members, two substances, the acid and the carbinol, rotate in one direction and the halide in the other. Second, the 1,1-methylethylpropionic acid (3) and the two derivatives are levorotatory whereas the higher 1,1-disubstituted propionic acids are dextrorotatory as is also the carbinol derived from it but the halide is levorotatory.

The 1,1-ethylpropylpropionic acid (3) similarly to the 1,1-methylethylpropionic acid (3) is levorotatory and the carbinol and the bromide are also levorotatory whereas all the higher acids of the series are dextrorotatory, as are the carbinols and halides.

In Table II a fourth column is introduced, the higher members of which have not yet been prepared but the directions of rotations of which may be predicted from the rotations of the members of the preceding columns.

Examining the part of Table II which contains the members of the 1,1-disubstituted propionic acids, we observe a series of squares drawn by light lines which contain the symmetric members of each series. Above these symmetric members are located substances which are enantiomorphous to some members of the preceding columns. Parallel to the symmetric line follows a line of squares enclosed in heavier lines. All the substances in these squares are levorotatory. Strictly speaking, the substances contained in these squares are the first members of the new series inasmuch as the members above the symmetric ones are enantiomorphous to the configurationally related group of substances. Thus

there must be a reason for the exceptional behavior of the first members. What is it?

If, roughly speaking, it be assumed that the rotation of each substance containing one asymmetric carbon atom is made up of two components, one part furnished by the radicle containing the polar group and the second by the arrangement of all the radicles attached to the asymmetric carbon, then an explanation can be found for the exceptional behavior of the first members of each series by one of two possible assumptions. First, it may be argued that the first members of each series possess the least asymmetry, as there is in them the least difference in weight of the two non-polar aliphatic radicles. On the other hand, the polar radicles remain the same in the entire series. If it is assumed that in the group of substances given in Table II the radicle containing the polar group contributes the levorotatory element and the non-polar radicle the dextrorotatory element, and if the second assumption is made that the levorotatory element remains within certain limits constant, and that the values of the non-polar radicle increase progressively from the first member, then the reason for the levorotation of the first member will become self-evident; namely, in it the numerical value of the dextrorotatory element is lower than that of the levorotatory. Furthermore, it will be easy to explain the levorotation of the halides of the higher members of the second column, the reason being that the radicle containing the halogen has the highest levorotation, as is seen from the higher levorotation of the methylethyl bromo-*n*-propylmethane, as compared with the rotations of the corresponding carbinol and the corresponding acid.

The higher members of the derivatives of the 1,1-disubstituted propionic acids (3) of the third column all show dextrorotation whereas the 1,1-ethylpropylpropionic acid and the corresponding carbinol and halides are all levorotatory. Thus again it can be concluded that the numerical value of the levorotatory element is higher than the dextrotatory in the first members and lower in the higher members. The fact that halides also are dextrorotatory may indicate that in a relative sense in this series in the halides also the numerical value of the dextrorotatory element is higher than the levorotatory. The second assumption postulates that the non-polar radicles

$$\begin{array}{c} CH_3 \\ | \\ HC- \\ | \\ C_2H_5 \end{array} \quad \text{and} \quad \begin{array}{c} C_2H_5 \\ | \\ HC- \\ | \\ C_3H_7 \end{array}$$

contribute a levorotation. Thus in the members enclosed in squares with heavy lines both contributions are levorotatory and hence the total rotations of the substances are levorotatory.

Respective Rotations of Members of the Disubstituted Acetic Acids and Corresponding 1,1-Disubstituted Propionic Acids (3)

Upon comparison of the rotation of the corresponding derivatives of the two series, it is observed that the acids of the corresponding members of the two series differ in the directions of their rotations. The principal difference in the structure of the substances is the presence of a CH_2 group between the carboxyl and the asymmetric carbon atom in the series of the 1,1-disubstituted propionic acids. Thus in the latter series, the carboxyl is at a greater distance from the asymmetric carbon atom and this difference in distance may be held responsible for the difference in effect on the rotation of the polar group. Similar effects of the distance on the rotation have been observed in the case of double bonds and more recently in the respective rotations of the secondary carbinols containing either an isopropyl or an isobutyl group.

SUMMARY

1. The change of rotation on substitution of the hydroxyl of the disubstituted 2,2-ethanols and of the 3,3-propanols (1) by a halogen is in the direction opposite to that of the carbinols. This observation substantiates the conclusion reached by Levene and Mikeska to the effect that dextrorotatory aliphatic secondary carbinols are configurationally related to the levorotatory secondary halides.

2. The exceptional behavior of the derivatives of 1,1-methylethylpropionic acid (3) and of 1,1-ethylpropylpropionic acid (3) is explained on the basis of the assumption that the rotation of each optically active substance may be regarded as the resultant of two components, both levorotatory, or of which one may be dextrorotatory and the other levorotatory.

3. Some radicles may function either as the dextrorotatory or the levorotatory elements depending upon their distance from the asymmetric carbon, as for instance, the carboxyl in Series I and II.

EXPERIMENTAL

Dextro-3-Methyl-1-Pentanol—A Grignard reagent was formed from 35 gm. of magnesium in ether and 210 gm. of 1-bromo-2-methylbutane, $[\alpha]_D^{29} = +1.59°$ (from 2-methyl-1-butanol, $[\alpha]_D^{29} = -2.72°$). 75 gm. of paraformaldehyde were added and the mixture stirred overnight. The Grignard reagent was decomposed with ice and hydrochloric acid and the carbinol extracted with ether. B.p. 80° at 47 mm.; yield, 90 gm.; $n_D^{25} = 1.4178$; D $\frac{27}{4}$ = 0.822.

$$[\alpha]_D^{27} = \frac{+1.37°}{1 \times 0.822} = +1.67°. \quad [M]_D^{27} = +1.70° \text{ (homogeneous)}$$

2.805 mg. substance: 7.240 mg. CO_2 and 3.400 mg. H_2O.

$C_6H_{14}O$.	Calculated.	C 70.52, H 13.82
	Found.	" 70.40, " 13.56

Levo-2-Bromopentane—65 gm. of methylpropyl carbinol, $[\alpha]_D^{25} = +3.28°$, were cooled in ice and saturated with hydrogen bromide. The solution was heated on a steam bath 1 hour, cooled, and saturated again with hydrogen bromide. The aqueous layer was separated and the 2-bromopentane shaken with cold concentrated sulfuric acid, dilute sodium carbonate solution, then water. It was then fractionated. B.p. 117° at 760 mm.; yield, 85 gm.; D $\frac{25}{4}$ = 1.208.

$$[\alpha]_D^{25} = \frac{-7.06°}{1 \times 1.208} = -5.84°. \quad [M]_D^{25} = -8.83° \text{ (homogeneous)}$$

4.290 mg. substance: 6.330 mg. CO_2 and 2.875 mg. H_2O.

$C_5H_{11}Br$.	Calculated.	C 39.73, H 7.34
	Found.	" 40.24, " 7.50

Levo-2-Propylbutyric Acid (4) (β-Methyl Caproic Acid)—8 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 55 gm. of ethyl malonate were added. To this solution 50 gm. of 2-bromopentane, $[\alpha]_D^{25} = -5.84°$, were added. The product was refluxed 3 hours, then poured into water. The malonic ester was extracted

with ether and distilled. It was hydrolyzed by boiling 2 hours with 3 mols of potassium hydroxide in 80 per cent alcohol. The alcohol was evaporated and the potassium salt acidified with dilute sulfuric acid. The malonic acid was extracted with ether and dried with anhydrous sodium sulfate. The ether was evaporated and the residue heated in a metal bath at 190° until carbon dioxide ceased coming off. It was then distilled under reduced pressure. The distillate was dissolved in sodium carbonate solution and freed from impurities by extraction with ether. The carbonate solution was then acidified and the acid extracted with ether and fractionated. B.p. 112° at 16 mm.; yield, 18 gm.; $D\frac{26}{4} = 0.912$.

$$[\alpha]_D^{26} = \frac{-0.46°}{1 \times 0.912} = -0.50°. \quad [M]_D^{26} = -0.66° \text{ (homogeneous)}$$

4.710 mg. substance: 11.225 mg. CO_2 and 4.525 mg. H_2O.

$C_7H_{14}O_2$.	Calculated.	C 64.57, H 10.84
	Found.	" 64.99, " 10.75

Levo-3-Bromohexane—100 gm. of ethyl-*n*-propyl carbinol, $[\alpha]_D^{22} = +1.73°$, were converted into the bromide as described for 2-bromopentane. B.p. 142° at 760 mm.; $D\frac{22}{4} = 1.166$.

$$[\alpha]_D^{22} = \frac{-2.37°}{1 \times 1.166} = -2.03°. \quad [M]_D^{22} = -3.35° \text{ (homogeneous)}$$

5.572 mg. substance: 8.978 mg. CO_2 and 3.978 mg. H_2O.

$C_6H_{13}Br$.	Calculated.	C 43.64, H 7.94
	Found.	" 43.94, " 7.99

3-Propyl Valeric Acid (*5*)—60 gm. of 3-bromohexane, $[\alpha]_D^{22} = -2.03°$, were condensed with ethyl malonate as described for 2-propylbutyric acid (4). The acid obtained was completely racemized.

Dextro-3-Propyl Valeric Acid (*5*)—The inactive acid was prepared from 3-bromohexane and ethyl malonate. 228 gm. of the inactive acid were dissolved in 1 liter of hot acetone and 650 gm. of quinine were added. The solution was filtered and allowed to crystallize in a refrigerator at 0°. It required 2 days for crystallization. After five crystallizations the salt was decomposed with

10 per cent hydrochloric acid and the organic acid extracted with ether. B.p. 106° at 5 mm.; $D\frac{30}{4} = 0.911$; $n_D^{25} = 1.4287$.

$$[\alpha]_D^{30} = \frac{+1.69^\circ}{1 \times 0.911} = +1.86^\circ. \quad [M]_D^{30} = +2.67^\circ \text{ (homogeneous)}$$

4.375 mg. substance: 10.680 mg. CO_2 and 4.510 mg. H_2O.

$C_8H_{16}O_2$.	Calculated.	C 66.62, H 11.19
	Found.	" 66.58, " 11.53

Dextro-Ethyl Ester of 3-Propyl Valeric Acid (5)—65 gm. of 3-propyl valeric acid (5), $[\alpha]_D^{30} = +1.86^\circ$, were mixed with 150 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The mixture was heated 1 hour on a steam bath. Then the excess alcohol was distilled off. The residue was shaken with dilute sodium carbonate solution, extracted with ether, and distilled. B.p. 80° at 9 mm.; yield, 67 gm.; $D\frac{30}{4} = 0.866$; $n_D^{25} = 1.4183$.

$$[\alpha]_D^{30} = \frac{+0.51^\circ}{1 \times 0.866} = +0.59^\circ. \quad [M]_D^{30} = +1.02^\circ \text{ (homogeneous)}$$

6.075 mg. substance: 15.405 mg. CO_2 and 6.425 mg. H_2O.

$C_{10}H_{20}O_2$.	Calculated.	C 69.71, H 11.60
	Found.	" 69.16, " 11.83

Dextro-3-Ethyl-1-Hexanol—60 gm. of the ethyl ester of 3-propyl valeric acid (5), $[\alpha]_D^{30} = +0.59^\circ$, were added to 300 cc. of absolute alcohol and reduced by dropping into a suspension of sodium in boiling toluene.[1] B.p. 73° at 15 mm.; $D\frac{28}{4} = 0.829$; $n_D^{25} = 1.4323$.

$$[\alpha]_D^{28} = \frac{+0.41^\circ}{1 \times 0.829} = +0.49^\circ. \quad [M]_D^{28} = +0.64^\circ \text{ (homogeneous)}$$

This carbinol was purified through its half phthalic ester, but the rotation did not change.

5.225 mg. substance: 14.090 mg. CO_2 and 6.460 mg. H_2O.

$C_8H_{18}O$.	Calculated.	C 73.78, H 13.94
	Found.	" 73.55, " 13.83

Dextro-1-Chloro-3-Ethyl Hexane—10 gm. of 3-ethyl-1-hexanol, $[\alpha]_D^{28} = +0.49^\circ$, were cooled in ice and 40 gm. of thionyl chloride were added. The mixture was heated 1 hour on a steam bath,

then fractionated. B.p. 85° at 40 mm.; D $\frac{27}{4}$ = 0.879; n_D^{25} = 1.4335.

$$[\alpha]_D^{27} = \frac{+1.01^\circ}{1 \times 0.879} = +1.15^\circ. \quad [M]_D^{27} = +1.68^\circ \text{ (homogeneous)}$$

0.1559 gm. substance: 0.150 gm. AgCl (Carius).
$C_8H_{15}Cl$. Calculated. Cl 24.19. Found. Cl 23.80

Dextro-1-Bromo-3-Ethyl Hexane—To 10 gm. of 3-ethyl-1-hexanol, $[\alpha]_D^{28}$ = +0.49°, cooled in ice, 20 gm. of phosphorus tribromide were added. The product was heated ½ hour on a steam bath, then poured on ice. The oily layer was shaken with concentrated sulfuric acid, then water, and dried with anhydrous sodium sulfate. B.p. 94° at 35 mm.; yield, 9 gm.; D $\frac{22}{4}$ = 1.119.

$$[\alpha]_D^{22} = \frac{+1.14^\circ}{1 \times 1.119} = +1.02^\circ. \quad [M]_D^{22} = +1.97^\circ \text{ (homogeneous)}$$

Levo-3-Bromoheptane—130 gm. of ethyl-*n*-butyl carbinol, $[\alpha]_D^{22}$ = +7.37°, were converted into the bromide as described for 2-bromopentane. B.p. 79° at 40 mm.; yield, 193 gm.; D $\frac{22}{4}$ = 1.139.

$$[\alpha]_D^{22} = \frac{-10.42^\circ}{1 \times 1.139} = -9.15^\circ. \quad [M]_D^{22} = -16.38^\circ \text{ (homogeneous)}$$

4.505 mg. substance: 7.815 mg. CO_2 and 3.340 mg. H_2O.

$C_7H_{15}Br$.	Calculated.	C 46.93, H 8.45
	Found.	" 47.31, " 8.29

Levo-3-Butyl Valeric Acid (*5*)—To 26 gm. of sodium in 300 cc. of absolute alcohol were added 180 gm. of ethyl malonate and 193 gm. of 3-bromoheptane, $[\alpha]_D^{22}$ = −9.15°. The procedure was the same as described for 2-propylbutyric acid (4).[1] B.p. 130° at 12 mm.; yield, 71 gm.; D $\frac{22}{4}$ = 0.908.

$$[\alpha]_D^{22} = \frac{-1.22^\circ}{1 \times 0.908} = -1.35^\circ. \quad [M]_D^{22} = -2.13^\circ \text{ (homogeneous)}$$

3.165 mg. substance: 7.995 mg. CO_2 and 3.130 mg. H_2O.

$C_9H_{18}O_2$.	Calculated.	C 68.31, H 11.47
	Found.	" 68.89, " 11.06

Levo-Ethyl Ester of 3-Butyl Valeric Acid (5)—71 gm. of 3-butyl valeric acid (5), $[\alpha]_D^{22} = -1.34°$, were mixed with 200 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. Esterification was carried out as described for the ethyl ester of 3-propyl valeric acid (5). B.p. 107° at 25 mm.; yield, 73 gm.; $D\frac{22}{4} = 0.866$.

$$[\alpha]_D^{22} = \frac{-0.49°}{1 \times 0.866} = -0.56°. \quad [M]_D^{22} = -1.05° \text{ (homogeneous)}$$

4.312 mg. substance: 11.210 mg. CO_2 and 4.565 mg. H_2O.

$C_{11}H_{22}O_2$. Calculated. C 70.89, H 11.91
Found. " 70.90, " 11.84

Levo-3-Ethyl-1-Heptanol—60 gm. of ethyl ester of 3-butyl valeric acid (5), $[\alpha]_D^{22} = -0.56°$, were dissolved in 300 cc. of absolute alcohol. This solution was dropped into a suspension of 120 gm. of sodium in 600 cc. of toluene as described for 3-ethyl-1-hexanol. B. p. 101° at 16 mm.; yield, 33 gm. (after purification through its half phthalic ester); $D\frac{23}{4} = 0.834$.

$$[\alpha]_D^{23} = \frac{-0.55°}{1 \times 0.834} = -0.66°. \quad [M]_D^{23} = -0.95° \text{ (homogeneous)}$$

3.352 mg. substance: 9.170 mg. CO_2 and 4.175 mg. H_2O.

$C_9H_{20}O$. Calculated. C 74.89, H 13.98
Found. " 74.61, " 13.94

Levo-1-Bromo-3-Ethyl Heptane—To 30 gm. of 3-ethyl-1-heptanol $[\alpha]_D^{23} = -0.66°$, cooled in ice, 60 gm. of phosphorus tribromide were added. The mixture was heated 1 hour on a steam bath, then poured on ice. The oily layer was shaken with concentrated sulfuric acid, then with a sodium carbonate solution, and finally dried and fractionated. B.p. 90° at 15 mm.; yield, 35 gm.; $D\frac{23}{4} = 1.103$.

$$[\alpha]_D^{23} = \frac{-0.35°}{1 \times 1.103} = -0.32°. \quad [M]_D^{23} = -0.66° \text{ (homogeneous)}$$

5.015 mg. substance: 9.575 mg. CO_2 and 4.125 mg. H_2O.

$C_9H_{19}Br$. Calculated. C 52.15, H 9.25
Found. " 52.07, " 9.20

Levo-3-Bromooctane—120 gm. of ethyl-*n*-amyl carbinol, $[\alpha]_D^{22} = +6.82°$, were converted into the bromide as described for 2-bromopentane. B.p. 85° at 25 mm.; yield, 166 gm.; $D\frac{22}{4} = 1.097$.

$$[\alpha]_D^{22} = \frac{-12.11^\circ}{1 \times 1.097} = -11.04^\circ. \quad [M]_D^{22} = -21.32^\circ \text{ (homogeneous)}$$

5.934 mg. substance: 10.960 mg. CO_2 and 4.735 mg. H_2O.

$C_8H_{17}Br$.	Calculated.	C 49.72, H 8.88
	Found.	" 50.37, " 8.93

Levo-3-n-Amyl Valeric Acid (5)—23 gm. of sodium were dissolved in 200 cc. of absolute alcohol and 160 gm. of ethyl malonate and then 160 gm. of 3-bromooctane, $[\alpha]_D^{22} = -11.04^\circ$, were added to the solution. The acid was prepared and purified as described for 2-propylbutyric acid (4). B.p. 140° at 12 mm.; yield, 55 gm.; $D\frac{22}{4} = 0.899$.

$$[\alpha]_D^{22} = \frac{-1.50^\circ}{1 \times 0.899} = -1.67^\circ. \quad [M]_D^{22} = -2.87^\circ \text{ (homogeneous)}$$

2.759 mg. substance: 7.070 mg. CO_2 and 2.975 mg. H_2O.

$C_{10}H_{20}O_2$.	Calculated.	C 69.69, H 11.71
	Found.	" 69.89, " 12.06

Levo-Ethyl Ester of 3-n-Amyl Valeric Acid (5)—55 gm. of 3-*n*-amyl valeric acid (5) were dissolved in 150 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid added. Esterification was carried out as described for the ethyl ester of 3-propyl valeric acid (5). B.p. 104° at 12 mm.; yield, 59 gm.; $D\frac{22}{4} = 0.865$.

$$[\alpha]_D^{22} = \frac{-0.66^\circ}{1 \times 0.865} = -0.76^\circ. \quad [M]_D^{22} = -1.53^\circ \text{ (homogeneous)}$$

4.705 mg. substance: 12.460 mg. CO_2 and 5.010 mg. H_2O.

$C_{12}H_{24}O_2$.	Calculated.	C 71.93, H 12.08
	Found.	" 72.22, " 11.91

Levo-3-Ethyl-1-Octanol—59 gm. of ethyl ester of 3-*n*-amyl valeric acid (5), $[\alpha]_D^{22} = -0.76^\circ$, were dissolved in 250 cc. of absolute alcohol and this dropped into a suspension of 100 gm. of sodium in 500 cc. of boiling toluene. The reduction was carried out as described for 3-ethyl-1-hexanol. B.p. 110° at 15 mm.; yield, 30 gm.; $D\frac{22}{4} = 0.833$.

$$[\alpha]_D^{22} = \frac{-0.96^\circ}{1 \times 0.833} = -1.15^\circ. \quad [M]_D^{22} = -1.82^\circ \text{ (homogeneous)}$$

3.689 mg. substance: 10.220 mg. CO_2 and 4.675 mg. H_2O.
$C_{10}H_{22}O$. Calculated. C 75.85, H 14.02
Found. " 75.56, " 14.18

Levo-1-Bromo-3-Ethyl Octane—25 gm. of 3-ethyl-1-octanol, $[\alpha]_D^{22} = -0.61°$, were cooled in ice and 50 gm. of phosphorus tribromide were added. Bromination was completed as described for 1-bromo-3-ethyl heptane. B.p. 99° at 14 mm.; yield, 29 gm.; $D\frac{22}{4} = 1.079$.

$$[\alpha]_D^{22} = \frac{-0.66°}{1 \times 1.079} = -0.61°. \quad [M]_D^{22} = -1.35° \text{ (homogeneous)}$$

3.282 mg. substance: 6.550 mg. CO_2 and 2.670 mg. H_2O.
$C_{10}H_{21}Br$. Calculated. C 54.27, H 9.57
Found. " 54.43, " 9.10

Dextro-6-Ethyl-3-Nonanol—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 37 gm. of 1-chloro-3-ethyl hexane, $[\alpha]_D^{27} = +1.15°$. This was cooled in ice and 20 gm. of propionaldehyde slowly added. The Grignard reagent was decomposed and the carbinol extracted in the usual manner. B.p. 114° at 16 mm.; yield, 31 gm.; $D\frac{22}{4} = 0.830$.

$$[\alpha]_D^{22} = \frac{+0.27°}{1 \times 0.830} = +0.32°. \quad [M]_D^{22} = +0.56° \text{ (homogeneous)}$$

3.876 mg. substance: 10.863 mg. CO_2 and 4.936 mg. H_2O.
$C_{11}H_{24}O$. Calculated. C 76.65, H 14.05
Found. " 76.44, " 14.25

Dextro-4-Ethyl Nonane—30 gm. of 6-ethyl-3-nonanol, $[\alpha]_D^{22} = +0.32°$, were heated under reflux with 200 gm. of "constant boiling" hydriodic acid for 15 minutes. The iodide was extracted with ether and the ether evaporated under reduced pressure. The residue was mixed with 100 gm. of finely divided zinc and reduced by adding concentrated hydrochloric acid. The hydrocarbon was extracted and purified as described previously.[1] B.p. 77° at 20 mm.; yield, 8 gm.; $D\frac{23}{4} = 0.745$.

$$[\alpha]_D^{23} = \frac{+0.52°}{1 \times 0.745} = +0.70°. \quad [M]_D^{23} = +1.09° \text{ (homogeneous)}$$

3.649 mg. substance: 11.242 mg. CO_2 and 5.029 mg. H_2O.
$C_{11}H_{24}$. Calculated. C 84.51, H 15.49
Found. " 84.02, " 15.42

Levo-4-Ethyl-1-Nonanol—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 75 gm. of 3-ethyl-1-bromooctane, $[\alpha]_D^{22} = -0.69°$. To the Grignard reagent were added 15 gm. of paraformaldehyde and the mixture was stirred 20 hours. The Grignard reagent was decomposed and the carbinol worked up as usual. B.p. 127° at 15 mm.; yield, 20 gm.

$$[\alpha]_D^{23} = 0° \text{ (homogeneous)}$$

4.120 mg. substance: 11.644 mg. CO_2 and 5.215 mg. H_2O.
$C_{11}H_{24}O$. Calculated. C 76.66, H 14.05
Found. " 77.08, " 14.16

Levo-4-Ethyl Nonane—20 gm. of 4-ethyl-1-nonanol, $[\alpha]_D^{23} = 0°$, were brominated by 25 gm. of phosphorus tribromide. The halide was isolated as previously described. B.p. 122° at 15 mm.; yield, 24 gm.; $D\frac{22}{4} = 1.054$.

$$[\alpha]_D^{22} = \frac{+0.62°}{2 \times 1.054} = +0.29°. \quad [M]_D^{22} = +0.71° \text{ (homogeneous)}$$

24 gm. of halide were reduced by forming a Grignard reagent with 3 gm. of magnesium in dry ether. This was poured onto ice and the hydrocarbon purified as previously described. B.p. 77° at 20 mm.; yield, 7 gm.; $D\frac{23}{4} = 0.745$.

$$[\alpha]_D^{23} = \frac{\cdot -0.74°}{2 \times 0.745} = -0.50°. \quad [M]_D^{23} = -0.76° \text{ (homogeneous)}$$

2.834 mg. substance: 8.738 mg. CO_2 and 3.920 mg. H_2O.
$C_{11}H_{24}$. Calculated. C 84.51, H 15.49
Found. " 84.09, " 15.48

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CONFIGURATIONAL RELATIONSHIP OF HYDROCARBONS

II. OPTICAL ROTATIONS OF HYDROCARBONS OF THE NORMAL SERIES

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The simplest group of substances studied with regard to relationships of structure and of optical activity is that of the secondary aliphatic carbinols. In these substances only two variable radicles are present as can be seen from figure (I).

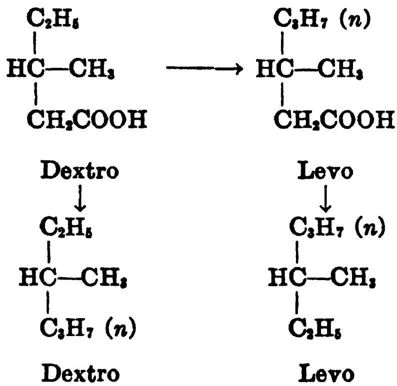
$$
\begin{array}{c}
R_1 \\
| \\
HCOH \\
| \\
R_2 \\
\\
\mathrm{I}
\end{array}
\qquad\qquad
\begin{array}{l}
CH_3 \\
\,| \\
HC{-}C_2H_5 \\
\,| \\
C_3H_7\ (n) \\
\\
\quad\mathrm{II}
\end{array}
$$

The simplest optically active hydrocarbon is a trisubstituted methane, namely methylethyl-*n*-propylmethane (II). From this hydrocarbon three different homologous series can be formed, depending upon whether the methyl, ethyl, or propyl group is substituted by a higher homologous radicle. In the present study only two of these series were investigated, namely the one derived by the progressive substitution of the group ethyl and the other derived by the progressive substitution of the group propyl. Also the series from the hydrocarbon (III) by progressive substitution of the group ethyl was studied.

$$
\begin{array}{l}
CH_3 \\
\,| \\
HC{-}C_2H_5 \\
\,| \\
C_4H_9\ (n) \\
\\
\quad\mathrm{III}
\end{array}
$$

It is evident that it was necessary to establish the configurational relationships of these three groups of hydrocarbons before they could be made use of for the purpose of discussing the relationship of chemical structure to optical activity. The task was no longer difficult inasmuch as the configurational relationship of disubstituted propionic acids has been established and inasmuch as the hydrocarbons can be derived directly from these acids.

In regard to the acids it will be recalled, in this place, that the dextro-1,1-methylethylpropionic acid (3) was correlated to levo-1,1-methylpropylpropionic acid (3) and that these two configurationally related acids lead to two enantiomorphous hydrocarbons.



I II

Thus one is confronted with the same question as in the case of secondary carbinols; namely, whether the configurationally related members of the methyl and of the ethyl series rotate in the same direction.

Should the general rule connecting the direction of rotation of the secondary carbinol with the structure be applicable to the hydrocarbons, then it is to be expected that all the members of the ethylmethyl series should remain dextrorotatory and that in the propylethyl series the members following the symmetric one should be dextrorotatory. Furthermore, it is to be expected that in the butylethyl series, the first two members should be levorotatory and the third symmetric and the one to the right of the symmetric should be dextrorotatory.

Hydrocarbons of three series were prepared by sets of reactions indicated in Table I. The rotations are summarized in Table II.

TABLE I

Experimental Values

$[M]_D^{25}$ (*in Degrees*

	—CHOH	—CH_2Br	—CH_2—COOH	—CH_2—CHOH—CH_3	—CH_2—CHOH—C_2H_5	—CH_2—CHOH—$C_3H_7(n)$	—$C_3H_7(n)$	—$C_4H_9(n)$	—$C_5H_{11}(n)$
CH_3	−2.13	+3.26	+4.25						
HC—									
C_2H_5	−1.97	+2.94		+4.25	+3.87	+5.28	+3.67	+5.07	+6.75

	—CH_2COOH	—CH_2—CH_2Br	—CH_2—CH_2—CHOH—CH_3	—CH_2—CH_2—CHOH—C_2H_5	—C_2H_5	—$C_3H_7(n)$	—$C_4H_9(n)$	—$C_5H_{11}(n)$
CH_3	+2.84*	−16.81*			+7.75			
HC—								
$C_3H_7(n)$	+1.28	−7.14	−0.46	−0.95		0	−0.59	−0.84
CH_3	+1.83	−5.44			+3.44		−0.57	−0.65
HC—								
$C_4H_9(n)$	+5.45*	−15.19*				+1.36		

* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

From Table II it can be seen that all members of the first series remain dextrorotatory. This was to be expected as in the entire

TABLE II

Calculated Maximum Rotations of Configurationally Related Hydrocarbons

From experimental values on the basis of C_2H_5–$\overset{|}{\mathrm{H}C}$–CH_3 with CH_2COOH (i.e. $C_2H_5\text{—}HC(CH_3)\text{—}CH_2COOH$) having a $[M]_D^{25} = +10.35°$.

C_2H_5 $HC\text{—}CH_3$ CH_2COOH + 10.35°					
C_2H_5 $HC\text{—}CH_3$ CH_2OH − 5.18°	C_2H_5 $HC\text{—}CH_3$ CH_2Br + 7.91°	C_2H_5 $HC\text{—}CH_3$ C_2H_5 0	C_2H_5 $HC\text{—}CH_3$ C_3H_7 (*n*) + 9.87°	C_2H_5 $HC\text{—}CH_3$ C_4H_9 (*n*) + 13.63°	C_2H_5 $HC\text{—}CH_3$ C_5H_{11} (*n*) + 18.16°
C_3H_7 (*n*) $HC\text{—}CH_3$ CH_2COOH − 3.60°	C_3H_7 (*n*) $HC\text{—}CH_3$ CH_2CH_2Br + 20.06°	C_3H_7 (*n*) $HC\text{—}CH_3$ C_2H_5 − 9.87°	C_3H_7 (*n*) $HC\text{—}CH_3$ C_3H_7 (*n*) 0	C_3H_7 (*n*) $HC\text{—}CH_3$ C_4H_9 (*n*) + 1.67°	C_3H_7 (*n*) $HC\text{—}CH_3$ C_5H_{11} (*n*) + 2.36°
C_4H_9 (*n*) $HC\text{—}CH_3$ CH_2COOH − 7.26°	C_4H_9 (*n*) $HC\text{—}CH_3$ CH_2CH_2Br + 20.20°	C_4H_9 (*n*) $HC\text{—}CH_3$ C_2H_5 − 13.63°	C_4H_9 (*n*) $HC\text{—}CH_3$ C_3H_7 (*n*) − 1.81°	C_4H_9 (*n*) $HC\text{—}CH_3$ C_4H_9 (*n*) 0	C_4H_9 (*n*) $HC\text{—}CH_3$ C_5H_{11} (*n*) + 0.86°
C_5H_{11} (*n*) $HC\text{—}CH_3$ CH_2COOH − 11.84°*	C_5H_{11} (*n*) $HC\text{—}CH_3$ CH_2CH_2Br + 21.61°	C_5H_{11} (*n*) $HC\text{—}CH_3$ C_2H_5 − 18.16°	C_5H_{11} (*n*) $HC\text{—}CH_3$ C_3H_7 (*n*) (− 2.36°)†	C_5H_{11} (*n*) $HC\text{—}CH_3$ C_4H_9 (*n*) (− 0.86°)†	C_5H_{11} (*n*) $HC\text{—}CH_3$ C_5H_{11} (*n*) 0

* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

† Not experimental values but those of the enantiomorphous hydrocarbons.

series the heaviest radicle remains in the same position. In the other series the members to the left of the symmetric one are levorotatory. In these the upper radicle is heavier; the members to the right of the symmetric one are dextrorotatory inasmuch as in them the lower radicle is the heavier. Thus the direction of rotation of hydrocarbons seems to be determined by the respective allocation of the heavier group as was the case with the secondary carbinols.

Numerical Values—Several hydrocarbons had been previously prepared from active amyl iodide by the Wurtz method of synthesis. Thus, Marckwald[1] prepared methylethylpropylmethane, Welt[2] prepared methylethylpropyl-, methylethylbutyl-, methylethylamyl-, and methylethylisoamylmethane. The molecular rotations calculated from the recorded specific rotations were practically identical for all the members of this homologous series. *A priori*, this finding did not seem very probable. Indeed, from the results reported in this communication, it may be concluded that the substances previously prepared were impure. From Table II it can be seen that in every homologous series the values of the molecular rotation increase progressively. According to the rule of Tschugaeff, a maximum value should be reached. As yet our series have not been carried far enough to enable us to say when such a maximum is reached in the case of hydrocarbons.

It must be stated that the values given in Table II are calculated values. Inasmuch as all the reactions are accomplished without racemization it was possible to compute the values of the rotation of all substances on the basis of the maximum rotation of the 1,1-methylethylpropionic acid (3). It is interesting to note that the numerical values of the rotation of the two enantiomorphous methylpropylbutylmethanes, prepared on one hand from 1,1-methylpropylpropionic acid (3) and on the other from 1,1-methylbutylpropionic acids (3), are practically the same, thus indicating that the method of calculation employed was reliable.

Referring again to the numerical values of the rotations of the hydrocarbons given in Table II, it can be seen that those of the second row and in the third column are much higher than those given in the other rows and columns. This point will be made the subject of a separate discussion.

[1] Marckwald, W., *Ber. chem. Ges.*, **13**, 37, 1046 (1880).

[2] Welt, I., *Compt. rend. Acad.*, **119**, 745 (1894).

SUMMARY

1. The hydrocarbons of several homologous series have been prepared.

2. The rule which has been formulated by Levene and Haller in regard to the direction of the rotation of the secondary carbinols is applicable also to optically active hydrocarbons.

3. The maximum values of the rotation of the hydrocarbons have been calculated indirectly. Evidence is furnished to show that the method of calculation is reliable.

EXPERIMENTAL

Levo-2-Methyl Butanol-1—The inactive carbinol was resolved by recrystallizing the brucine salt of its half phthalic ester from acetone. After ten crystallizations the rotation of the phthalate was

$$[\alpha]_D^{23} = \frac{+0.25° \times 100}{1 \times 21.7} = +1.15° \text{ (in absolute alcohol)}$$

The brucine salt was decomposed with dilute hydrochloric acid and the phthalate hydrolyzed by boiling with potassium hydroxide. The carbinol was then distilled. B.p. 127° at 760 mm.; $D\frac{28}{4}$ = 0.816.

$$[\alpha]_D^{23} = \frac{-1.97°}{1 \times 0.816} = -2.41°. \quad [M]_D^{23} = -2.12° \text{ (homogeneous)}$$

Dextro-1-Bromo-2-Methyl Butane—50 gm. of 2-methyl butanol-1, $[\alpha]_D^{23} = -2.41°$, were cooled in ice and 100 gm. of phosphorus tribromide were slowly added. The product was let stand overnight at room temperature, then heated on a steam bath for 10 minutes. It was poured on ice and the oily layer separated. This was shaken with cold concentrated sulfuric acid then washed with dilute sodium carbonate solution, dried, and distilled. B.p. 119° at 760 mm.; $D\frac{22}{4}$ = 1.218.

$$[\alpha]_D^{22} = \frac{+2.63°}{1 \times 1.218} = +2.16°. \quad [M]_D^{22} = +3.26° \text{ (homogeneous)}$$

The rotation obtained for this bromide is considerably higher than that previously reported in the literature. However, if the

directions for preparation, which are reported in the literature, are followed a much lower rotation is obtained due to racemization.

Dextro-2-Ethylbutyric Acid (*4*)—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 35 gm. of 1-bromo-2-methyl butane, $[\alpha]_D = +2.16°$. A stream of dry carbon dioxide was passed through the reagent for about 15 minutes. This Grignard solution was decomposed in the usual manner. The ether was evaporated and the residue dissolved in sodium hydroxide solution and extracted with ether. The alkaline solution was acidified and the organic acid extracted with ether and distilled. B.p. 110° at 30 mm.; yield 27 gm.; $D\frac{22}{4} = 0.925$.

$$[\alpha]_D^{22} = \frac{+3.39°}{1 \times 0.925} = +3.66°. \quad [M]_D^{22} = +4.25° \text{ (homogeneous)}$$

3.460 mg. substance: 7.937 mg. CO_2 and 3.249 mg. H_2O.
$C_6H_{12}O_2$. Calculated. C 62.01, H 10.42
Found. " 62.55, " 10.50

Dextro-3-Methyl Hexanol-5—A Grignard reagent was prepared from 12 gm. of magnesium turnings in 500 cc. of dry ether and 77 gm. of 1-bromo-2-methyl butane, $[\alpha]_D^{22} = +1.95°$ (from 2-methyl butanol, $[\alpha]_D = -2.24°$). The solution was cooled in ice and 30 gm. of acetaldehyde slowly run in with stirring. The Grignard solution was decomposed in the usual manner and the carbinol distilled. B.p. 146–147° at 760 mm.; yield 42 gm.; $D\frac{22}{4} = 0.816$.

$$[\alpha]_D^{22} = \frac{+3.46°}{1 \times 0.816} = +4.24°. \quad [M]_D^{22} = +4.92° \text{ (homogeneous)}$$

3.114 mg. substance: 8.260 mg. CO_2 and 3.870 mg. H_2O.
$C_7H_{16}O$. Calculated. C 72.33, H 13.89
Found. " 72.33, " 13.90

Dextro-3-Methyl Hexane—12 gm. of 3-methyl hexanol-5, $[\alpha]_D^{22} = +4.24°$, were distilled twice from 100 gm. of saturated hydriodic acid solution. The iodide was not isolated. This was added to an excess of zinc turnings and reduced by stirring and dropping in concentrated hydrochloric acid. After reduction was complete the hydrocarbon was extracted with ether and the ether distilled. The residue was purified by shaking with cold concentrated sulfuric acid followed by sodium carbonate solution. It was then

dried with anhydrous sodium sulfate and distilled from a small piece of sodium. B.p. 91–92° at 760 mm.; yield 5 gm.; $D\frac{22}{4}$ = 0.687.

$$[\alpha]^{22}_{D} = \frac{+\ 2.52^\circ}{1 \times 0.687} = +\ 3.67^\circ.\quad [M]^{22}_{D} = +\ 3.67^\circ \text{ (homogeneous)}$$

Calculated maximum rotation: $[\alpha]^{22}_{D}$ = +9.87°. $[M]^{22}_{D}$ = +9.87° (homogeneous)

3.614 mg. substance: 11.145 mg. CO_2 and 5.170 mg. H_2O.

C_7H_{16}.	Calculated.	C 83.89, H 16.11
	Found.	" 84.09, " 15.99

Dextro-3-Methyl Heptanol-5—This carbinol was prepared from 8 gm. of magnesium in dry ether, 53 gm. of 1-bromo-2-methyl butane, $[\alpha]^{22}_{D}$ = +1.95°, and 25 gm. of propionaldehyde, as described for 3-methyl hexanol-5. B.p. 72° at 22 mm.; yield 32 gm.; $D\frac{23}{4}$ = 0.816.

$$[\alpha]^{23}_{D} = \frac{+\ 2.43^\circ}{1 \times 0.816} = +\ 2.98^\circ.\quad [M]^{23}_{D} = +\ 3.88^\circ \text{ (homogeneous)}$$

2.760 mg. substance: 7.480 mg. CO_2 and 3.510 mg. H_2O.

$C_8H_{18}O$.	Calculated.	C 73.76, H 13.94
	Found.	" 73.90, " 14.23

Dextro-3-Methyl-5-Bromoheptane—30 gm. of 3-methyl heptanol-5, $[\alpha]^{23}_{D}$ = +2.98°, were cooled in ice and treated with 60 gm. of phosphorus tribromide. This carbinol was very hard to brominate and was heated 2 hours on a steam bath. The bromide was purified as described for 1-bromo-2-methyl butane. B.p. 62° at 16 mm.; $D\frac{22}{4}$ = 1.077.

$$[\alpha]^{22}_{D} = \frac{+\ 5.19^\circ}{1 \times 1.077} = +\ 4.82^\circ.\quad [M]^{22}_{D} = +\ 9.30^\circ \text{ (homogeneous)}$$

4.675 mg. substance: 8.559 mg. CO_2 and 3.825 mg. H_2O.

$C_8H_{17}Br$.	Calculated.	C 49.73, H 8.88
	Found.	" 49.98, " 9.15

Dextro-3-Methyl Heptane—A Grignard reagent was prepared from 5 gm. of powdered magnesium in ether and 20 gm. of 3-methyl-5-bromoheptane, $[\alpha]^{22}_{D}$ = +4.82°. The Grignard reagent was stirred and warmed for 15 minutes after final addition of the

halide. It was then poured on ice and hydrochloric acid and the hydrocarbon extracted with ether. The ether was evaporated and the hydrocarbon purified as described for 3-methyl hexane. B.p. 116–118° at 760 mm.; $D\frac{22}{4} = 0.710$.

$$[\alpha]_D^{22} = \frac{+3.16°}{1 \times 0.710} = +4.45°. \quad [M]_D^{22} = +5.08° \text{ (homogeneous)}$$

Calculated maximum rotation: $[\alpha]_D^{22} = +11.97°$. $[M]_D^{22} = +13.63°$ (homogeneous)

2.705 mg. substance: 8.373 mg. CO_2 and 3.780 mg. H_2O.

C_8H_{18}.	Calculated.	C 84.10, H 15.90
	Found.	" 84.13, " 15.87

Dextro-3-Methyl Octanol-5—This carbinol was prepared from 8 gm. of magnesium in dry ether, 53 gm. of 2-methyl-1-bromobutane, $[\alpha]_D^{22} = +1.95°$, and 30 gm. of *n*-butylaldehyde as described for 3-methyl hexanol-5. B.p. 89° at 15 mm.; yield 43 gm.; $D\frac{23}{4} = 0.822$.

$$[\alpha]_D^{23} = \frac{+3.02°}{1 \times 0.822} = +3.67°. \quad [M]_D^{23} = +5.30° \text{ (homogeneous)}$$

3.526 mg. substance: 9.708 mg. CO_2 and 4.340 mg. H_2O.

$C_9H_{20}O$.	Calculated.	C 74.91, H 13.98
	Found.	" 75.08, " 13.77

Dextro-3-Methyl-5-Bromooctane—43 gm. of 3-methyl octanol-5, $[\alpha]_D^{23} = +3.67°$, were cooled in ice and 60 gm. of phosphorus tribromide were added. The product was heated, ½ hour on a steam bath, then poured on ice. The halide was purified as described for 1-bromo-2-methyl butane. B.p. 94° at 20 mm.; yield 47 gm.; $D\frac{23}{4} = 1.054$.

$$[\alpha]_D^{23} = \frac{+5.93°}{1 \times 1.054} = +5.63°. \quad [M]_D^{23} = +11.65°$$

4.608 mg. substance: 8.862 mg. CO_2 and 3.810 mg. H_2O.

$C_9H_{19}Br$.	Calculated.	C 52.16, H 9.25
	Found.	" 52.44, " 9.25

Dextro-3-Methyl Octane—A Grignard reagent was prepared from 6 gm. of magnesium in ether and 45 gm. of 3-methyl-5-bromooctane, $[\alpha]_D^{23} = +5.63°$. This was poured on ice and the hydro-

carbon extracted and purified as described for 3-methyl heptane. B.p. 143–144° at 760 mm.; yield 9 gm. (after purification); $D\frac{23}{4}$ = 0.725.

$$[\alpha]^{23}_{D} = \frac{+\ 3.82^\circ}{1 \times 0.725} = +\ 5.27^\circ. \quad [M]^{23}_{D} = +\ 6.75^\circ \text{ (homogeneous)}$$

Calculated maximum rotation $[\alpha]^{23}_{D} = +14.18^\circ$. $[M]^{23}_{D} = +18.16^\circ$ (homogeneous)

3.675 mg. substance: 11.371 mg. CO_2 and 5.185 mg. H_2O.

C_9H_{20}.	Calculated.	C 84.27, H 15.73
	Found.	" 84.37, " 15.79

Levo-5-Methyl-2-Octanol—A Grignard reagent was prepared from 8 gm. of magnesium in 200 cc. of dry ether and 60 gm. of 1-bromo-3-methyl hexane, $[\alpha]^{21}_{D} = -3.99^\circ$. To this reagent were added 15 gm. of acetaldehyde in ether. The Grignard solution was decomposed and the carbinol obtained in the usual manner. B.p. 92° at 15 mm.; yield 35 gm.; $D\frac{25}{4}$ = 0.821.

$$[\alpha]^{25}_{D} = \frac{-\ 0.52^\circ}{2 \times 0.821} = -\ 0.32^\circ. \quad [M]^{25}_{D} = -\ 0.46^\circ \text{ (homogeneous)}$$

3.955 mg. substance: 10.870 mg. CO_2 and 4.875 mg. H_2O.

$C_9H_{20}O$.	Calculated.	C 74.91, H 13.98
	Found.	" 74.94, " 13.79

Levo-5-Methyl Octane—35 gm. of 5-methyl-2-octanol, $[\alpha]^{25}_{D}$ = -0.32°, were distilled twice from 200 gm. of hydriodic acid, sp. gr. 1.70. The crude iodide was not purified. This was placed in a 1 liter flask with 200 gm. of zinc turnings and reduced by adding slowy 500 cc. of concentrated hydrochloric acid. It was placed on a steam bath when the initial reaction subsided, until the zinc was dissolved. The hydrocarbon was extracted with ether and the ether distilled. The crude hydrocarbon was shaken with cold concentrated sulfuric acid, washed with sodium carbonate solution, then water, and dried with dry sodium sulfate. It was refluxed 1 hour with a small piece of metallic sodium and then fractionated. B.p. 53° at 25 mm.; yield 22 gm.; $D\frac{22}{4}$ = 0.714.

$$[\alpha]^{22}_{D} = \frac{-\ 0.65^\circ}{2 \times 0.714} = -\ 0.46^\circ. \quad [M]^{22}_{D} = -\ 0.58^\circ \text{ (homogeneous)}$$

3.491 mg. substance: 10.785 mg. CO_2 and 4.956 mg. H_2O.
C_9H_{20}. Calculated. C 84.27, H 15.73
Found. " 84.24, " 15.88

Levo-6-Methyl-3-Nonanol—This carbinol was prepared by the action of propionaldehyde on a Grignard reagent formed from 8 gm. of magnesium in ether and 60 gm. of 1-bromo-3-methyl hexane, $[\alpha]_D^{24} = -3.99°$. B.p. 105° at 15 mm.; yield 29 gm.; $D\frac{24}{4} = 0.820$.

$$[\alpha]_D^{24} = \frac{-0.53°}{1 \times 0.820} = -0.65°. \quad [M]_D^{24} = -1.02° \text{ (homogeneous)}$$

5.010 mg. substance: 13.855 mg. CO_2 and 6.260 mg. H_2O.
$C_{10}H_{22}O$. Calculated. C 75.86, H 14.02
Found. " 75.41, " 13.98

Levo-6-Methyl Nonane—29 gm. of 6-methyl-3-nonanol, $[\alpha]_D^{24} = -0.65°$, were distilled twice with 200 gm. of hydriodic acid, sp. gr. 1.70. The iodide was separated and reduced by zinc and hydrochloric acid as described for 5-methyl octane. B.p. 72° at 25 mm.; yield 8 gm.; $D\frac{22}{4} = 0.731$.

$$[\alpha]_D^{22} = \frac{-0.86°}{2 \times 0.731} = -0.59°. \quad [M]_D^{22} = -0.84° \text{ (homogeneous)}$$

4.485 mg. substance: 13.880 mg. CO_2 and 6.240 mg. H_2O.
$C_{10}H_{22}$. Calculated. C 84.40, H 15.60
Found. " 84.39, " 15.57

Levo-6-Methyl-3-Decanol—This carbinol was prepared by the action of 25 gm. of propionaldehyde on the Grignard reagent formed from 12 gm. of magnesium in ether and 80 gm. of 1-bromo-3-methyl heptane, $[\alpha]_D^{24} = -2.79°$. The carbinol was isolated in the usual way. B.p. 117° at 22 mm.; yield 35 gm.; $D\frac{24}{4} = 0.829$.

$$[\alpha]_D^{24} = \frac{-0.54°}{2 \times 0.829} = -0.33°. \quad [M]_D^{24} = -0.56° \text{ (homogeneous)}$$

4.990 mg. substance: 14.045 mg. CO_2 and 6.085 mg. H_2O.
$C_{11}H_{24}O$. Calculated. C 76.66, H 14.05
Found. " 76.75, " 13.65

Levo-6-Methyl Decane—30 gm. of 6-methyl-3-decanol, $[\alpha]_D^{24} = -0.33°$, were heated with 200 gm. of hydriodic acid, sp. gr. 1.70,

until the iodide was formed. The oily layer as separated and reduced by zinc and concentrated hydrochloric acid as described for 5-methyl octane. B.p. 94° at 30 mm.; yield 8 gm.; D $\frac{24}{4}$ = 0.738.

$$[\alpha]_D^{24} = \frac{-0.58^\circ}{2 \times 0.738} = -0.39^\circ. \quad [M]_D^{24} = -0.61^\circ \text{ (homogeneous)}$$

3.410 mg. substance: 10.541 mg. CO_2 and 4.820 mg. H_2O.
$C_{11}H_{24}$. Calculated. C 84.51, H 15.49
Found. " 84.29, " 15.81

Dextro-3-Methyl Heptane—A Grignard reagent was prepared from 6 gm. of magnesium in ether and 50 gm. of 1-bromo-3-methyl heptane, $[\alpha]_D^{24} = -2.79^\circ$. The Grignard reagent was poured on ice and the hydrocarbon was isolated and purified as described for 5-methyl octane. B.p. 117–118° at 760 mm.; yield 14 gm.; D $\frac{22}{4}$ = 0.710.

$$[\alpha]_D^{22} = \frac{+2.24^\circ}{1 \times 0.710} = +3.15^\circ. \quad [M]_D^{22} = +3.60^\circ$$

3.852 mg. substance: 11.865 mg. CO_2 and 5.465 mg. H_2O.
C_8H_{18}. Calculated. C 84.10, H 15.90
Found. " 84.00, " 15.87

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CONFIGURATIONAL RELATIONSHIP OF HYDROCARBONS

III. THE OPTICAL ROTATIONS OF THE HYDROCARBONS OF THE SERIES METHYLISOBUTYLMETHANE

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Previous observations in this laboratory[1] have led to the conclusion that in simple substances of the types I and II, the direction of rotation

$$\begin{array}{ccc} & R_1 & \\ & | & \\ H- & C & -OH \\ & | & \\ & R_2 & \\ & & \\ & \text{I} & \end{array} \qquad \begin{array}{ccc} & R_1 & \\ & | & \\ H- & C & -CH_3 \\ & | & \\ & R_2 & \\ & & \\ & \text{II} & \end{array}$$

is determined by the respective weights of the groups R_1 and R_2. When R_1 is smaller in weight than R_2, the substances of type I rotate to the right. If R_1 contains a polar group, however, the direction of rotation is determined by the distance of the polar group from the asymmetric carbon atom and not by the weights of the radicles, unless the polar group is very far removed from the asymmetric carbon atom.

Later, on the basis of indirect evidence, it was concluded that when R_1 contains an isopropyl group, then again the direction of rotation of a substance is determined by the distance of the isopropyl group from the asymmetric carbon atom and unless the isopropyl group is very far removed from the asymmetric carbon atom, the weights of the other radicles play a subordinate part in their effect on the direction of rotation.

On the basis of these considerations, and on the basis of the

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 91, 405, 761 (1931).

observations on the rotations of the secondary carbinols of the isobutyl series, it was expected that configurationally related hydrocarbons of the homologous series

$$(i)\quad \begin{array}{c} C_4H_9 \\ | \\ H—C—CH_3 \\ | \\ R \end{array}$$

TABLE I*

Maximum Molecular Rotations of Configurationally Related Hydrocarbons Calculated on Basis of Maximum Rotation of Acids from Which They Were Prepared

$[M]^{25}_{D}$

	$—CH_2COOH$† (1)	$—C_2H_5$ (2)	$—n\text{-}C_3H_7$ (3)	$—n\text{-}C_4H_9$ (4)	$—n\text{-}C_5H_{11}$ (5)	$—CH_2—CH(CH_3)—CH_3$ (6)
$C_2H_5—CH(CH_3)—$	+10.35	0	+9.9	+11.4	+12.0	+21.3
$n\text{-}C_3H_7—CH(CH_3)—$	−3.60	−9.9	0	+1.7	+2.4	+14.9
$n\text{-}C_4H_9—CH(CH_3)—$	−6.06	−11.4	−1.5	0	+0.8	+11.9
$n\text{-}C_5H_{11}—CH(CH_3)—$	−8.12	−12.5	−2.4	−0.8	0	+9.3

* On reexamination it was found that the methylethyl-*n*-butyl and methylethyl-*n*-amylmethanes previously described contained traces of the unsaturated hydrocarbons. The repurified methylethyl-*n*-amylmethane was found to have the rotation given in this table. The methyl-*n*-butylpropionic and methyl-*n*-amylpropionic acids were then resolved to maximum and converted into the hydrocarbons. Therefore, the values given in this *Journal* (Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 764 (1931)) should be corrected according to this table.

† See Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 761 (1931).

would all rotate in the same direction regardless of the molecular weight of the radicle R; furthermore, it was expected that the numerical values of the rotation of the individual members would be in descending order, the member with R equals C_2H_5 having the highest rotation.

A series of hydrocarbons of this general type has now been prepared. In Table I, Column 6, are summarized the results of observations on the hydrocarbons of this series. In Columns 2 to 5 are given the rotations of the hydrocarbons of the normal series. In the latter the rotations of the carbinols situated above the symmetric member rotate in opposite direction from those below. The direction of rotation of the members of the isobutyl series does not change and is of descending order, as was expected. Thus, the direction of rotation of simple substances can in a way be predicted on the basis of earlier observations.

TABLE II

Experimental Values for Molecular Rotations of Configurationally Related Hydrocarbons Containing an Isobutyl Group

	$-CH_2COOH$	$-CH{=}C(CH_3)_2$	$-CH_2-CH(CH_3)_2$
$C_2H_5-CH(CH_3)-$	−6.0		−12.4
n-$C_3H_7-CH(CH_3)-$	+1.1	−7.7	−4.5
n-$C_4H_9-CH(CH_3)-$	+1.8	−5.0	−3.6
n-$C_5H_{11}-CH(CH_3)-$	+2.5	−4.8	−2.8

The configurational relationships of the hydrocarbons here discussed have been established on the basis of the method of preparation. They have been prepared from disubstituted 1,1-propionic acid (3) of known configurational relationship.

The maximum values given in Table I are derived by computation on the basis of the knowledge of the maximum rotation of the disubstituted acids.

The experimental values for molecular rotations of configurationally related hydrocarbons containing an isobutyl group are given in Table II.

EXPERIMENTAL

2,4-Dimethyl Heptanol-2—To 1 mol of methyl magnesium iodide in ether were added 60 gm. of ethyl ester of 2-propyl butyric acid-4 (from 2-propyl butyric acid-4, $[M]_D^{24} = +1.08°$). The Grignard reagent was decomposed in the usual manner and the carbinol distilled. B.p. 134–136° at 760 mm., yield 33 gm.

Levo-2,4-Dimethyl Heptane—33 gm. of 2,4-dimethyl heptanol-2 (above) were mixed with 10 gm. of oxalic acid and distilled. Dehydration took place very readily. The unsaturated hydrocarbon which collected in the receiver was separated from the water, dried with sodium sulfate, then distilled from a small piece of metallic sodium. B.p. 134–136° at 760 mm., yield 25 gm., $D\frac{24}{4} = 0.739$.

$$[\alpha]_D^{24} = \frac{-4.51°}{1 \times 0.739} = -6.10°. \quad [M]_D^{24} = -7.69° \text{ (homogeneous)}$$

3.562 mg. substance: 11.045 mg. CO_2 and 4.540 mg. H_2O.

C_9H_{18}.	Calculated.	C 85.62, H 14.38
	Found.	" 84.55, " 14.26

The unsaturated hydrocarbon was mixed with 0.5 gm. of platinic oxide catalyst and reduced by shaking with hydrogen under a pressure of 30 pounds per square inch. The reduction took only 15 minutes for completion. The hydrocarbon was separated from the catalyst and shaken with cold concentrated sulfuric acid. It was washed with sodium carbonate solution, followed by water, and dried with dry sodium sulfate. It was then distilled from a small piece of metallic sodium. B.p. 131–131.5° at 760 mm., yield 19 gm., $D\frac{22}{4} = 0.733$.

$$[\alpha]_D^{22} = \frac{-2.57°}{1 \times 0.733} = -3.51°. \quad [M]_D^{22} = -4.49° \text{ (homogeneous)}$$

5.521 mg. substance: 17.140 mg. CO_2 and 7.585 mg. H_2O.

C_9H_{20}. Calculated.	C 84.27,	H 15.73
Found.	" 84.65,	" 15.39

2,4-Dimethyl Octanol-2—This was prepared from 1 mol of methyl magnesium iodide and 60 gm. of ethyl ester of 2-butyl butyric acid-4 (from 2-butyl butyric acid-4, $[M]_D^{24} = +1.83°$). The carbinol was isolated in the usual way. Due to its instability it was distilled, but not further purified.

Levo-2,4-Dimethyl Octane—The carbinol from the above preparation was mixed with 10 gm. of oxalic acid and distilled. The unsaturated hydrocarbon was separated from the water, dried with dry sodium sulfate, then distilled from a small piece of metallic sodium. B.p. 62° at 30 mm., yield 27 gm., $D\,\frac{24}{4} = 0.743$.

$$[\alpha]_D^{24} = \frac{-2.67°}{1 \times 0.743} = -3.59°. \quad [M]_D^{24} = -5.04° \text{ (homogeneous)}$$

4.150 mg. substance: 13.030 mg. CO_2 and 5.370 mg. H_2O.

$C_{10}H_{20}$. Calculated.	C 85.61,	H 14.39
Found.	" 85.61,	" 14.53

20 gm. of the unsaturated hydrocarbon were mixed with 0.5 gm. of platinic oxide and reduced by hydrogen under a pressure of 30 pounds per square inch. The hydrocarbon was purified as described for 2,4-dimethyl heptane. B.p. 70° at 40 mm., yield 17 gm., $D\,\frac{24}{4} = 0.725$.

$$[\alpha]_D^{24} = \frac{-1.82°}{1 \times 0.725} = -2.51°. \quad [M]_D^{24} = -3.57° \text{ (homogeneous)}$$

4.150 mg. substance: 12.870 mg. CO_2 and 5.790 mg. H_2O.

$C_{10}H_{22}$. Calculated.	C 84.40,	H 15.60
Found.	" 84.56,	" 15.61

2,4-Dimethyl Nonanol-2—This was prepared from 1 mol of methyl magnesium iodide and 60 gm. of ethyl ester of 2-amyl butyric acid-4 (from 2-amyl butyric acid-4, $[M]_D^{24} = +2.47°$). The carbinol was isolated and distilled as previously described but not purified further.

Levo-2,4-Dimethyl Nonane—The carbinol above was mixed with 10 gm. of oxalic acid and distilled. The unsaturated hydrocarbon was separated from the water, dried with dry sodium sulfate, and distilled from metallic sodium. B.p. 79° at 30 mm., $D\,\frac{24}{4} = 0.751$.

$$[\alpha]_D^{24} = \frac{-\ 2.32^\circ}{1 \times 0.751} = -\ 3.09^\circ. \quad [M]_D^{24} = -\ 4.76^\circ \text{ (homogeneous)}$$

3.935 mg. substance: 12.295 mg. CO_2 and 5.040 mg. H_2O.

$C_{11}H_{22}$.	Calculated.	C 85.62, H 14.38
	Found.	" 85.20, " 14.33

20 gm. of the unsaturated hydrocarbon were mixed with 0.5 gm. of platinic oxide and reduced by hydrogen under a pressure of 30 pounds per square inch. The hydrocarbon was isolated and purified as described for 2,4-dimethyl heptane. B.p. 75° at 25 mm., yield 18 gm., D $\frac{24}{4}$ = 0.731.

$$[\alpha]_D^{24} = \frac{-\ 1.33^\circ}{1 \times 0.731} = -\ 1.82^\circ. \quad [M]_D^{24} = -\ 2.84^\circ \text{ (homogeneous)}$$

3.812 mg. substance: 11.835 mg. CO_2 and 5.270 mg. H_2O.

$C_{11}H_{24}$.	Calculated.	C 84.51, H 15.49
	Found.	" 84.66, " 15.47

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SYNTHETIC NUCLEOSIDES*

III. THEOPHYLLINE-*d*-GLUCODESOSIDE

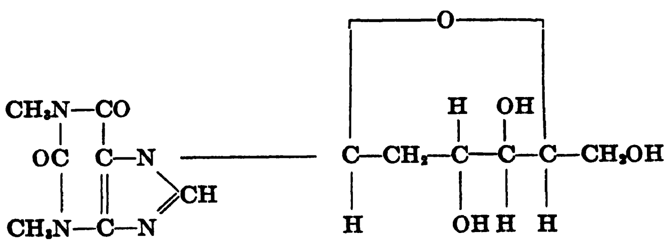
BY P. A. LEVENE AND FRANK CORTESE

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, April 13, 1931)

The further knowledge of the details of the structure of thymus nucleic acid depends, in the first place, upon information regarding the details of the structure of the ribodesosenucleosides entering into its structure. However, the wider study of the structure of these substances is beset with many difficulties, as the naturally occurring substances are obtainable in small quantities only. Furthermore, the usual methods of determining the ring structure of glucosides were found ineffective when applied to nucleosides. It was thought, therefore, that the synthetic method might offer a better approach to the elucidation of those details in the structure of the nucleosides which as yet remain unknown.

Methods have been worked out for the preparation of nucleosides of normal sugars. Whether the same methods would lead to the synthesis of ribodesosenucleosides was not certain, particularly in view of the great instability of the desosides. The present communication contains a report on the synthesis of theophylline-*d*-glucodesoside. This was accomplished through the condensation of the silver salt of theophylline with 1-bromo-3,4,6-tribenzoyl-*d*-glucodesose which on saponification led to the desired nucleoside of the following structure.



* The first two publications in this series are: Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 65, 463, 469 (1925).

The details of the synthesis and of the physical properties of this substance are given in the experimental part. Work on the synthesis of ribodesosides is in progress.

EXPERIMENTAL

Tribenzoyl Theophylline-d-Glucodesoside—8.1 gm. of pure benzobromoglucodesose,[1] previously dried over phosphorus pentoxide for 1 hour under reduced pressure, were dissolved in 180 cc. of dry benzene. 9 gm. of the silver salt of theophylline, dried to constant weight at 130° under reduced pressure, were added and the mixture was boiled 10 minutes. The completion of the reaction was shown by the absence of a precipitate, when a drop of the reaction solution was shaken with a silver nitrate solution, acidified with nitric acid. The silver bromide was filtered and the almost colorless hot filtrate was treated with charcoal for 1 minute. Filtration now gave a colorless solution. On standing overnight at room temperature, 0.1 gm. of theophylline was deposited. The filtrate from this was poured into 2 liters of petroleum ether (b.p. 30–40°) and the whole thoroughly stirred. The fluffy white precipitate was allowed to settle and was washed twice by decantation with the precipitant. The flask was then put on the steam bath and acetone added. As the ether boiled away, more acetone was added, until complete solution occurred. 500 cc. of hot absolute alcohol were added, after concentration to a small volume. This was, in turn, concentrated until crystallization set in. 750 cc. of hot absolute alcohol were stirred in and the flask was stored overnight in the ice box. The solid mass of tiny hair-like silky needles was filtered, washed with petroleum ether, and air-dried several hours. The yield was 4.7 gm. or 73 per cent based on the benzobromoglucodesose.

This product contains no solvent of crystallization. It has no decisive melting point. It will shrink at about 140°, soften at about 150°, and the turbid melt will become clear between 170–193°, when effervescence also occurs. The behavior is probably due to gradual decomposition on heating. If the product is recrystallized from hot chloroform and excess cold ether, and the resulting needles are air-dried several hours, it will then show a

[1] Bergmann, M., Schotte, H., and Leschinsky, W., *Ber. chem. Ges.*, **56**, 1055 (1923).

melting point of 122°. This is fairly consistent. However, the crystals now have about 8 per cent solvent, and if this be driven off at 80° under reduced pressure, the crystals will then give the same ragged and unsatisfactory melting point as the original product. Recrystallization from boiling pyridine and excess cold ether also furnishes a product melting at about 122°. Vacuum drying of this again restores the original ragged melting point.

The product was recrystallized twice from chloroform and ether. The crystals were dried to constant weight at 80° under reduced pressure.

Rotation and Analysis

$$[\alpha]_D^{22} = \frac{+0.33° \times 100}{1 \times 2.32} = +14.2° \text{ (in } s\text{-tetrachloroethane)}$$

4.421 mg. substance: 10.355 mg. CO_2 and 1.945 mg. H_2O.
4.949 " " : 0.382 cc. N_2 (756 mm., 24°).

$C_{34}H_{30}N_4O_9$.	Calculated.	C 63.95, H 4.70, N 8.78
	Found.	" 63.87, " 4.92, " 8.82

Tribenzoyl theophylline-*d*-glucodesoside is insoluble in water, ethyl alcohol, ether, and petroleum ether. It is soluble in acetone, benzene, chloroform, and pyridine. It crystallizes in needles, which form in rosettes.

Theophylline-d-Glucodesoside—The usual methods of hydrolysis with ammonia fail to split off the benzoyl groups from tribenzoyl theophylline-*d*-glucodesoside. A more drastic method had to be resorted to, and a rather poor yield of the nucleoside was obtained.

3.5 gm. of tribenzoyl theophylline-*d*-glucodesoside were suspended in a filtered solution of 2.8 gm. of crystalline barium hydroxide in 75 cc. of anhydrous methyl alcohol. After 2 minutes boiling, complete solution had taken place. A strong ester odor indicated that the benzoyl groups had been split off to form methyl benzoate. As refluxing continued, a heavy precipitation gradually occurred. After 1 hour, the odor of methyl benzoate had disappeared. The flask was cooled under the tap, and the mixture of theophylline-*d*-glucodesoside and barium benzoate was filtered, washed with methyl alcohol, ether, and air-dried. The mixture amounted to 3.4 gm. The colorless methyl alcoholic filtrate was stored overnight in the ice box and yielded 0.2 gm. of theophylline-*d*-glucodesoside in small colorless cubes and rectangles.

The mixture of nucleoside and barium benzoate was triturated with 15 cc. of cold water to remove the major portion of the latter. The residue (1.5 gm.) was dissolved in hot water and the solution cooled to 0°.

Sulfuric acid was cautiously added to a faint excess. After filtering the mixture of barium sulfate and benzoic acid, ammonia was added to a slight excess to prevent any possible subsequent hydrolysis. The solution was now evaporated to dryness under reduced pressure in a bath maintained at 35–40°. The residue amounted to 0.9 gm. This was triturated with 2 N ammonium hydroxide, filtered, and washed with 2 N ammonium hydroxide, water, acetone, and ether. This procedure removed the ammonium sulfate and ammonium benzoate. The residue of pure nucleoside amounted to 0.3 gm.

The total yield was, therefore, 0.5 gm. or about 30 per cent.

The nucleoside was recrystallized from hot water and acetone, into tiny cubes and rectangles, which contained no water of crystallization. The cubes were actually rhombs with almost equal axes. It melted at 258° without decomposition. The presence of a slight amount of impurity markedly affected the melting point. A sample with 0.3 per cent ash melted at 203° with decomposition. Another slightly impure sample melted at 251° with decomposition but the micro analysis of the sample detected no ash, and the carbon, hydrogen, and nitrogen values were excellent.

Rotation and Analysis

$$[\alpha]_D^{23} = \frac{-0.28° \times 100}{1 \times 1.04} = -26.9° \text{ in water}$$

4.103 mg. substance: 7.237 mg. CO_2 and 1.930 mg. H_2O.
5.355 " " : 0.792 cc. N_2 (752 mm., 24°).

4.170 mg. substance: 7.298 mg. CO_2 and 2.070 mg. H_2O.
4.195 " " : 0.628 cc. N_2 (752 mm., 24°).

$C_{13}H_{18}N_4O_6$.	Calculated.	C 47.85, H 5.52, N 17.18
	Found.	" 48.09, " 5.26, " 16.82
		" 47.72, " 5.52, " 17.02

The nucleoside is only slightly soluble in cold water, but soluble in hot water and in hot pyridine. It is insoluble in methyl and

ethyl alcohols, ether, petroleum ether, acetone, benzene, and chloroform. It is tasteless. It is stable in boiling Fehling's solution. A 1 minute boiling with 2 N hydrochloric acid causes hydrolysis, as shown by subsequent reduction of Fehling's solution. The nucleoside is apparently stable toward boiling with 0.05 N hydrochloric acid for 1 minute.

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ACETYL MONOSES

VII. THE ISOMERIC TRIACETYL-1-METHYL-*d*-RIBOSIDES

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(Received for publication, April 23, 1931)

Fischer, Bergmann, and Rabe[1] have observed that on "methylation" of 1-bromotriacetyl rhamnose, a triacetylmethyl-*l*-rhamnoside is obtained, characterized by possessing one acetyl group stable towards alkali. Dale[2] found that 1-bromotetracetyl-*d*-mannosė behaved similarly and Levene and Wolfrom[3] found the same peculiarity in the case of lyxose. These anomalous forms were referred to as γ forms. Levene and Wolfrom[4] have found that the rate of hydrolysis of the methyl group in the cases of γ-tetracetylmethyl-*d*-mannoside and of γ-triacetylmethyl-*d*-lyxoside resembles that of the furanosides, but Haworth and coworkers[5] have shown that in the cases of rhamnose and mannose, the glycoside possesses the pyranose structure. These authors concluded also that the stable acetyl group is situated on carbon atom 2. On the other hand, Freudenberg and Braun,[6] on the basis of spectroscopic analysis, reached the conclusion that the substance was not a true methylglycoside but that its carbon atoms 1 and 2 were linked to a methylated orthoacetic acid residue. Haworth and coworkers evolved a similar theory and structure independently and practically simultaneously.

[1] Fischer, E., Bergmann, M., and Rabe, A., *Ber. chem. Ges.*, **53**, 2362 (1920).

[2] Dale, J. K., *J. Am. Chem. Soc.*, **46**, 1046 (1924).

[3] Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **78**, 525 (1928).

[4] Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **79**, 471 (1928).

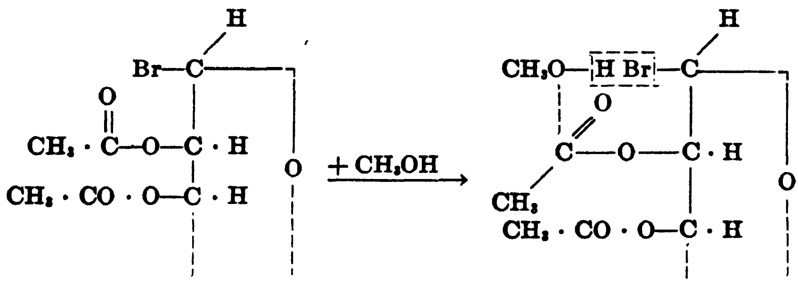
[5] Haworth, W. N., Hirst, E. L., and Miller, E. J., *J. Chem. Soc.*, 2469 (1929). Bott, H. G., Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 1395 (1930).

[6] Freudenberg, K., and Braun, E., *Naturwissenschaften*, **18**, 393 (1930).

As yet it is not known whether the peculiarity observed in these three sugars is common to all sugars having the hydroxyls of carbon atoms 2 and 3 in the *cis* position and because of this uncertainty the behavior of ribose has been made the subject of the present investigation.

It was found that 1-bromotriacetyl-*d*-ribose behaved similarly to the bromoacetyl derivatives of the other three sugars in forming a γ-triacetylmethyl-*d*-riboside which was obtained in crystalline form, with a m.p. of 77–78° and having $[\alpha]_D^{26} = +2.4°$ (in chloroform). The methyl group was hydrolyzed by dilute acids as readily as in the other γ-acetylmethylmonosides, but hydrolysis by alkali only revealed the presence of two acetyl groups. The substance was distinctly different in its properties from the triacetylmethyl-*d*-riboside obtained on acetylation of methyl-*d*-riboside, which acted as a normal, pyranose derivative.

Assuming that the formation of derivatives of orthoacetic acid is a common property of sugars having the hydroxyls of carbon atoms 2 and 3 in the *cis* position, this behavior may possibly be ascribed to the bromine atom of the bromoacetyl derivative of *d*-ribose having the α-configuration, and of those of *l*-rhamnose, *d*-lyxose, and *d*-mannose having the β-configuration (*i.e.*, the functional groups of carbon atoms 1 and 2 are also in the *cis* position).



EXPERIMENTAL

Preparation of Tetracetyl Ribose—5 gm. of crystalline ribose were dissolved in a mixture of 19 cc. of acetic anhydride and 25 cc. of pyridine and the resulting solution was kept at 0° overnight. It was then poured onto finely crushed ice, with vigorous stirring, and the crystalline precipitate filtered off. On standing overnight at 0° the aqueous mother liquor deposited a further crop of

crystalline material. The aqueous solution was now extracted three times with chloroform and the chloroform extract washed successively with ice-cold dilute sulfuric acid, ice-cold dilute sodium bicarbonate solution, and finally with ice water until neutral. It was then dried by means of anhydrous sodium sulfate, filtered, and the filtrate evaporated to a thick syrup. This was dissolved in 95 per cent ethyl alcohol and on nucleating and cooling, deposited crystalline tetracetyl ribose.

The total yield (after one recrystallization from 95 per cent ethyl alcohol) was 7.5 gm.; m.p., 110°.

Its rotation was

$$[\alpha]_D^{24} = \frac{-3.08° \times 100}{2 \times 2.960} = -52.0° \text{ (in chloroform)}$$

The substance had the following composition.

3.500 mg. substance: 6.305 mg. CO_2 and 1.830 mg. H_2O.

$C_{13}H_{18}O_9$.	Calculated.	C 49.04,	H 5.7
	Found.	" 49.12,	" 5.9

Preparation of Crystalline Bromotriacetyl Ribose—5 gm. of finely powdered tetracetyl ribose were mixed with 25 cc. of glacial acetic acid containing 40 per cent of dry hydrogen bromide. The resulting solution was allowed to stand at room temperature for 60 minutes, after which the hydrogen bromide gas was removed under diminished pressure at room temperature. The solution was then diluted with 100 cc. of toluene and evaporated to a thick syrup under diminished pressure at 35°. Two further portions of 50 cc. of toluene were run in and evaporated off. This syrup was now dissolved in 50 cc. of benzene and the solution was evaporated to a thick syrup. Traces of solvent were removed at high vacuum at 35°.

The resulting thick, very pale yellow syrup was dissolved in the minimum of cold dry ether, a further 5 cc. of ether were added, and then petroleum ether was added to incipient turbidity. A little charcoal was added and the mixture shaken and filtered on a fluted filter, the filtrate obtained being absolutely colorless.

On cooling in ice and scratching vigorously, crystallization immediately set in. Yield, 3.1 to 3.2 gm. of colorless crystals; m.p., 96°.

Its rotation was

$$[\alpha]_D^{25} = \frac{-7.55° \times 100}{2 \times 1.804} = -209.3° \text{ (in chloroform)}$$

The substance had the following composition.

8.150 mg. substance: 4.606 mg. AgBr (direct precipitation).
$C_{11}H_{15}O_7Br$. Calculated. Br 23.58. Found. Br 24.05

By the action of dry silver acetate upon crystalline bromotriacetyl ribose dissolved in toluene, the original tetracetyl ribose (m.p., 110°) was regenerated. After recrystallization from 95 per cent ethyl alcohol, its rotation was

$$[\alpha]_D^{25} = \frac{-0.90° \times 100}{2 \times 0.828} = -54.3° \text{ (in chloroform)}$$

Action of Methyl Alcohol (in Presence of Silver Carbonate) upon Bromotriacetyl Ribose—30 cc. of dry methyl alcohol were added quickly to an intimate mixture of 2.5 gm. of finely powdered, crystalline bromotriacetyl ribose with 6 gm. of dry, freshly prepared silver carbonate and the mixture vigorously shaken for 30 minutes, after which time no bromine was found in a small filtered test portion. The mixture was filtered, the silver salts well washed with dry ether, and the combined filtrate and washings evaporated to a thick syrup under diminished pressure at room temperature. It was dissolved in dry ether and the small amount of pink, flocculent silver precipitate removed by adding a little charcoal and filtering. The filtrate was allowed to evaporate slowly in a vacuum desiccator to a colorless syrup which crystallized spontaneously. It was recrystallized from a mixture of dry ether and petroleum ether at −15°, being obtained as long rectangular plates, m.p. 77–78°. Yield (after one recrystallization), 0.495 gm.

Its rotation was

$$[\alpha]_D^{26} = \frac{+0.05° \times 100}{2 \times 1.025} = +2.4° \text{ (in chloroform)}$$

The substance had the following composition.

4.305 mg. substance: 7.845 mg. CO_2 and 2.391 mg. H_2O.
6.200 " " : 4.890 " AgI.

$C_{12}H_{18}O_8$.	Calculated.	C 49.64,	H 6.3,	OMe 10.69	
	Found.	" 49.69,	" 6.2,	" 10.42	

100 mg. substance required 7.33 cc. 0.1 N NaOH (alkaline hydrolysis).

$C_8H_{12}O_6\cdot(CH_3CO)_2$.	Calculated.	$COCH_3$	29.7	(for 2 hydrolyzable acetyl groups)
	Found.	"	31.5	

A comparison of the rate of hydrolysis of this substance with those of the γ forms of tetracetyl methylmannoside and triacetylmethyllyxoside[4] was made. A solution of 100 mg. of substance in 5 cc. of absolute ethyl alcohol was prepared, and this was diluted to 10 cc. with 0.02 N aqueous hydrochloric acid. The rotation was observed immediately. The solution was then heated in a sealed glass tube at 98° for 90 minutes after which the rotation of the cooled solution was again observed. It was strongly reducing towards boiling Fehling's solution.

$$[\alpha]_{D}^{22} = \frac{+0.36^\circ \times 100}{2 \times 1.01} = +17.8^\circ \text{ (initial)}$$

$$[\alpha]_{D}^{22} = \frac{-0.39^\circ \times 100}{2 \times 1.01} = -19.3^\circ \text{ (final)}$$

Under the same conditions, the *normal* triacetyl methylriboside showed no change in rotation, indicating its relatively greater stability in the presence of 0.01 N hydrochloric acid (alcoholic). The specific rotation both before and after heating in a sealed tube at 98° for 90 minutes was, in this case,

$$[\alpha]_{D}^{25} = \frac{-0.37^\circ \times 100}{2 \times 1.445} = -12.8^\circ$$

The solution was very faintly reducing to boiling Fehling's solution on prolonged boiling.

Preparation of Normal Triacetyl Methylriboside—Polarimetric observations on a 1 per cent solution of crystalline ribose in 1 per cent methyl alcoholic hydrogen chloride at 27° showed that the specific rotation rapidly changed from $[\alpha]_{D}^{27} = -14.7^\circ$ (2 minutes after admixture) to a maximum value $[\alpha]_{D}^{27} = -2.4^\circ$ (14 minutes)

and thereafter decreased to an apparently constant value $[\alpha]_D^{27} = -41.5$ (56 hours).

Accordingly, 5 gm. of ribose were dissolved in 50 gm. of cold 1.5 per cent methyl alcoholic hydrogen chloride and kept at room temperature (23°) until the maximum value had been passed (40 minutes). It was boiled gently under a reflux for a further 105 minutes after which it was cooled in ice, shaken with a little charcoal, filtered, and the rotation of the filtrate observed. $[\alpha]_D^{23} = -39.5°$. The hydrogen chloride was now neutralized with silver carbonate, the mixture filtered, and the silver salts extracted several times with boiling methyl alcohol under a reflux. The combined filtrate was evaporated to a thick syrup under diminished pressure at 35°. Slight deposition of silver salt occurred so the syrup was redissolved in methyl alcohol, shaken with a little charcoal, filtered, and again evaporated to a thick syrup (yield, 5.4 gm.). All attempts to obtain either the α or the β form of methylriboside from this mixture were unsuccessful.[7]

5 gm. of syrupy methylriboside were dissolved in a mixture of 20 cc. of acetic anhydride and 25 cc. of pyridine, with cooling in ice. The solution was kept at 0° for 16 hours after which it had assumed a jelly-like consistency. It was poured onto crushed ice with vigorous stirring but, even on standing, only a negligible amount of insoluble gum was deposited. The aqueous liquor was therefore extracted three times with chloroform and the extract washed successively with ice-cold dilute sulfuric acid, ice-cold dilute sodium bicarbonate solution, and ice water until neutral. It was then dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to a thick syrup under diminished pressure at 35°. All attempts to crystallize the product were of no avail. Yield, 8.5 gm. of syrup.

This was distilled at high vacuum giving a main fraction (weight, 5.2 gm.) boiling at 120° at 0.05 mm. (bath temperature, 140°). This was a very pale yellow, fairly viscous syrup having $N_D^{24} = 1.4523$ and

$$[\alpha]_D^{24} = \frac{-1.00° \times 100}{2 \times 2.879} = -17.4° \text{ (in chloroform)}$$

[7] Levene, P. A., Jacobs, W. A., and Medigreceanu, F., *J. Biol. Chem.*, 11, 371 (1912).

Its composition was as follows:

5.991 mg. substance: 10.975 mg. CO_2 and 3.391 mg. H_2O.
5.185 " " : 4.150 " AgI.

$C_{12}H_{18}O_8$. Calculated.	C 49.64, H 6.3, OMe 10.69	
Found.	" 49.95, " 6.3, " 10.57	

100 mg. syrupy substance required 10.22 cc. 0.1 N NaOH (alkaline hydrolysis).

$C_6H_9O_5\cdot(CH_3CO)_3$. Calculated. $COCH_3$ 44.5 (for 3 hydrolyzable acetyl groups)
Found. " 44.0

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THE RING STRUCTURE OF DIACETONE GALACTOSE

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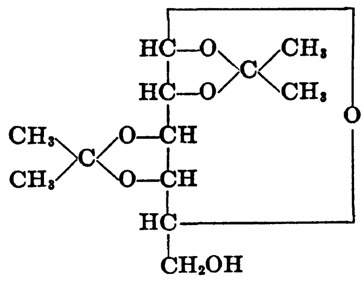
(Received for publication, May 28, 1931)

The diacetone derivatives of aldohexoses and aldopentoses so far examined all belong to the furanose type with the exception of diacetone arabinose of which the ring structure has not yet been proved by the direct method. Also, in the case of diacetone galactose the ring structure is not yet known. *A priori*, however, the pyranose structure may be expected for diacetone galactose inasmuch as the hydroxyl in position (6) in this derivative was shown by Svanberg and Bergmann[1] and by Freudenberg and coworkers[2] to be unsubstituted, and also inasmuch as in this case the hydroxyls in positions (3) and (4) offer the most favorable condition for the formation of a strainless 5-membered isopropylidene ring. This may be seen from the following figure.



Indeed, Freudenberg expressed preference for this structure.

Several years ago[3] we came into possession of monoacetone

[1] Svanberg, O., and Bergmann, S. W., *Arkiv. Kemi, Mineral. Geol.*, **9**, 3 (1924).

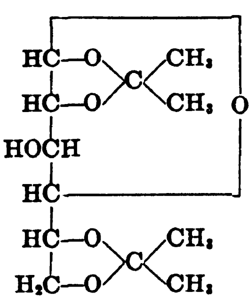
[2] Freudenberg, K., and Smeykal, K., *Ber. chem. Ges.*, **59**, 100 (1926). Freudenberg, K., and Raschig, K., *Ber. chem. Ges.*, **60**, 1633 (1927).

[3] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **64**, 473 (1925).

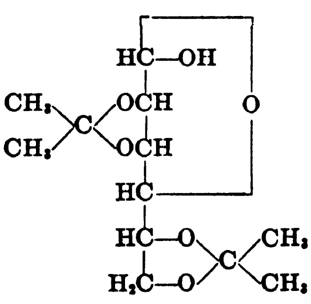
galactose which was formed spontaneously in the preparation of diacetone galactose. However, the quantity then in our possession was insufficient for an exhaustive study of its structure. A somewhat larger quantity of the substance has now been prepared by a process described in the experimental part, and this material was used for the determination of its ring structure. The substance was methylated in acetonic solution with dimethyl sulfate and sodium hydroxide. The trimethylmonoacetone-*d*-galactose prepared in this manner was oxidized to the corresponding acid and this converted into the lactone. On the basis of Hudson's rule the <1, 4> lactone should be levorotatory and the <1, 5> lactone dextrorotatory. The one obtained by us had a rotation of $[\alpha]_D^{20} = +46.8°$, thus indicating the <1, 5> ring structure of the lactone. This conclusion has been substantiated by the rate of the lactone formation of the free acid, inasmuch as half equilibrium was reached in 6 hours. Thus it is definitely established that the monoacetone-*d*-galactose belongs to the pyranose type and hence it is certain that diacetone-*d*-galactose likewise belongs to this type.

In view of the analogy in the configurations of galactose and arabinose it may be warranted to assume that diacetone arabinose also belongs to the pyranose type.

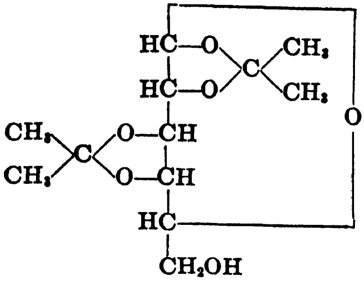
By comparison of the diacetone derivatives of three hexoses and of two pentoses, it can be seen that in every case the ring is formed in such a way as to facilitate acetonylation.



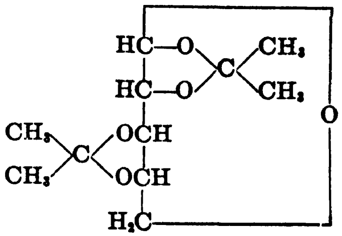
Diacetone-*d*-glucose



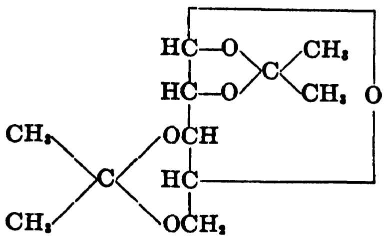
Diacetone-*d*-mannose



Diacetone-*d*-galactose



Diacetone-*l*-arabinose



Diacetone-*d*-xylose

EXPERIMENTAL

Diacetone-d-Galactose—A mixture of 100 gm. of galactose, 200 gm. of anhydrous copper sulfate, 2000 cc. of commercial acetone, and 10 cc. of concentrated sulfuric acid was shaken at room temperature for 18 to 20 hours. The acetone solution was filtered and neutralized with dry calcium hydroxide. The filtered solution was concentrated under diminished pressure to a syrup and distilled. The yield of diacetone galactose boiling at 131–135°, p = 0.2 mm., was 96 gm.

Monoacetone-d-Galactose—Large quantities of diacetone galactose, admixed with varying small amounts of hydrochloric acid in acetone, were allowed to stand in the cold room (temperature 10–16°) during the summer months. The best results were obtained when 100 gm. of diacetone galactose were diluted with a small amount of acetone and stirred with 1 cc. of 2 per cent hydrochloric acid in acetone. A total of about 25 gm. of recrystallized monoacetone galactose (with m.p. 157°) was obtained.

Trimethylmonoacetone-d-Galactose—5 gm. of galactose mono-

acetone, admixed with 15 cc. of acetone, were methylated with 45 cc. of dimethyl sulfate and 100 cc of 33 per cent sodium hydroxide at 55–70°. The reaction product was extracted with chloroform, the solution dried with anhydrous sodium sulfate, and the solvent removed under reduced pressure. Trimethylmonoacetone galactose was obtained as a colorless, fairly mobile liquid, distilling at 105°, p = 0.3 mm. It does not reduce Fehling's solution.

Its optical rotation was

$$[\alpha]_D^{20} = \frac{-1.35° \times 100}{1 \times 4.306} = -31.4° \text{ (in methyl alcohol)}$$

The substance had the following composition.

4.606 mg. substance: 9.240 mg. CO_2 and 3.435 mg. H_2O.
4.900 " " : 13.095 " AgI.

$C_{12}H_{22}O_6$.	Calculated.	C 54.96,	H 8.46,	OMe	35.5
	Found.	" 54.74,	" 8.34,	"	35.2

Trimethyl-d-Galactose—5 gm. of trimethylmonoacetone galactose were heated at 70° for 2 hours with 50 cc. of 0.2 N sulfuric acid. The acid was neutralized with barium carbonate, the solution was filtered and concentrated under reduced pressure to a syrup which was taken up in ether and dried with anhydrous sodium sulfate, and the solvent was removed. No attempt was made to distil the free syrupy sugar. It was heated for several hours at about 80° under 0.1 mm. pressure. Yield 4.5 gm. It reduced boiling Fehling's solution. Its optical rotation was

$$[\alpha]_D^{20} = \frac{-0.18° \times 100}{1 \times 4.16} = -4.3° \text{ (in methyl alcohol)}$$

It had the following composition.

5.342 mg. substance: 9.500 mg. CO_2 and 3.960 mg. H_2O.
5.306 " " : 17.030 " AgI.

$C_9H_{18}O_6$	Calculated.	C 48.75,	H 8.11,	OMe	41.9
	Found.	" 48.49,	" 8.29,	"	42.3

Trimethyl-δ-Galactonolactone—3.2 gm. of trimethylmonoacetone galactose were heated at 70° with 25 cc. of 0.05 N hydrobromic acid for 2 hours. The reaction mixture was transferred to a distilling flask with an equal volume of water and the liquid concentrated to its original volume to remove the liberated acetone. Bromine was

now added in portions of 0.5 gm. (total 3.5 gm.) during 4 days, the flask being kept at 35–40°. After removal of the bromine by aeration, the hydrobromic acid was removed with silver oxide and the filtered solution was titrated exactly with dilute hydrochloric acid. The filtered solution was concentrated to a syrup and heated for 4 hours at 100° (0.1 mm.) to complete the lactonization.

It had the following composition.

4.601 mg. substance:	8.245 mg. CO_2 and 2.900 mg. H_2O.		
7.192 " "	: 22.965 " AgI.		
$C_6H_{10}O_6$.	Calculated.	C 49.06, H 7.28, OMe 42.30	
	Found.	" 48.86, " 7.05, " 42.14	

0.1036 gm. of lactone required for neutralization 4.71 cc. of 0.1 N NaOH (phenolphthalein as indicator). Calculated 4.71 cc. of 0.1 N NaOH.

The initial optical rotation of the lactone in water was

$$[\alpha]_D^{20} = \frac{+1.15° \times 100}{1 \times 2.456} = +46.8°$$

Sodium Salt—0.1036 gm. of the lactone was dissolved in 4.9 cc. of 0.1 N NaOH and made to 5 cc. with water. The rotation is calculated on the sodium salt.

$$[\alpha]_D^{25} = \frac{+0.72° \times 100}{1 \times 2.45} = +29.4°$$

Free Acid—0.2009 gm. of the lactone was allowed to stand for several hours with 4.8 cc. of 0.2 N NaOH and then neutralized with 4.8 cc. of 0.2 N HCl and made to a volume of 10 cc. The readings as taken in a 2 dm. tube are given in the following tabulation.

	α_D^{25}	$[\alpha]_D^{25}$
Initial	+0.10	2.5
60 min.	+0.20	5.0
2 hrs.	+0.25	6.3
5 "	+0.35	8.8
6 "	+0.40	10.0 (20 per cent lactone)
24 "	+0.65	16.2
48 "	+0.75	18.7
72 "	+0.80	20.0 (40 per cent lactone) constant

Addendum—When the procedure as outlined for the preparation of diacetone galactose (p. 171) is applied to glucose, the yield of diacetone glucose is close to 70 per cent of the theoretical. In addition, the whole procedure (including shaking) only requires 24 hours, thus representing a considerable saving of time as compared with previous methods.

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STROPHANTHIN

XX. The Conversion of Isostrophanthidic Acid into the Desoxo Derivative

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(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, March 9, 1931)

The cardiac aglucones, the formulations of which have now been definitely established, are as follows:

Strophanthidin	$C_{23}H_{32}O_6$
Digitoxigenin	$C_{23}H_{34}O_4$
Gitoxigenin	$C_{23}H_{34}O_5$
Digoxigenin[1]	$C_{23}H_{34}O_5$
Periplogenin	$C_{23}H_{34}O_5$
Sarmentogenin	$C_{23}H_{34}O_5$

The close structural relationship of these aglucones, which is at once superficially suggested by these formulations, has been more definitely emphasized by common features in structure which have come to light during their individual investigation. They have all been found to be polyhydroxytetracyclic-$\Delta^{\beta,\gamma}$-lactones which under the influence of alkali may be isomerized to saturated substances. The extent, however, to which these structural resemblances would be maintained remained a subject for further inquiry and one of great importance in the problem. If the individual aglucones could be separately correlated with strophanthidin, the structural investigation of the group would then be greatly simplified.

Strophanthidin differs from the other aglucones (with the exception of antiarigenin) by its possession of an aldehydic carbonyl

[1] Smith, S., *J. Chem. Soc.*, 509 (1930). Mannich, C., Mohns, P., and Mauss, W., *Arch. Pharm.*, **268**, 453 (1930).

group. By the conversion of strophanthidin into a desoxo derivative a substance of the formula $C_{23}H_{34}O_5$ would result, which might be directly compared with the above naturally occurring aglucones of the same formulation. For purposes of correlation, therefore, the transformation of this aldehyde group to methyl has been made the subject of careful study by us.

Strophanthidin itself was, however, found to be useless for this purpose because of its general susceptibility to most reagents, with the formation of obscure transformation products. We have turned, therefore, to certain derivatives of strophanthidin which appeared to be more suitable, namely isostrophanthidic acid[2] and the hydrogenated anhydrostrophanthidins. Our experience with the latter will be presented in a separate communication.

In the case of isostrophanthidic acid, it was found possible to accomplish the desired transformation into the desoxo derivative by the use of the method of Wolff, after the miscarriage of other methods. After heating the *semicarbazone* with sodium ethylate, a crystalline acid could be separated from the reaction mixture, although in poor yield; this was definitely shown to possess the formula $C_{23}H_{34}O_6$. This was confirmed by the analysis of its *methyl ester* and, as will be presented in the following paper, by its identification with *isoperiplogenic acid.*

Previous to the successful use of the method of Wolff we had attempted to apply the method of Clemmensen. When this was carried out in acetic acid solution by the usual procedure, only non-crystalline reaction products were obtained. When, however, methyl alcohol was used as the solvent, simultaneous esterification occurred and it was possible to obtain in rather poor yield a neutral crystalline substance. The analysis of this reaction product gave results which agreed with the formulation $C_{24}H_{36}O_7$. Investigation showed that the aldehyde group had been reduced to the primary alcoholic group.[3] Simultaneously, isomerization

[2] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 61, 387 (1924).

[3] The reduction of the aldehyde group to the CH_2OH group has already been described in the case of the formation of dihydrostrophanthidol (Jacobs, W. A., *J. Biol. Chem.*, 88, 528 (1930)). Our experience with the above aglucones has shown no evidence of the presence in them of such a primary alcoholic group although it is probable that such aglucones may be found in nature.

to the γ^4 series occurred under the influence of the conditions used, since the substance (γ-isostrophanthidolic methyl ester) was strongly dextrorotatory. When a modified Clemmensen reaction was performed at lower temperature, the resulting substance gave similar analytical figures but like α-isostrophanthidic acid and its ester proved to be weakly levorotatory ($[\alpha]_D = -14.0$) and was therefore *α-isostrophanthidolic methyl ester*.[5] On a previous occasion we have already noted the fact that α-isostrophanthidic acid itself, at least under the conditions used, apparently is not converted into a γ-isomer. This is very curious in view of the fact that the corresponding acid, α-isostrophanthic acid, and the primary alcohol now described undergo such isomerization.

The effort was made to replace the primary hydroxyl group of this reduction product by halogen with the hope of subsequent reduction of the resulting CH_2X group to methyl. But the usual reagents such as the halogen acids, the phosphorus halides, and thionyl chloride gave us no tangible result. In order to protect the possibly interfering hydroxyl groups already present in isostrophanthidic methyl ester, an attempt was made to acetylate them. By the direct use of acetyl chloride, however, only the secondary hydroxyl (OH^{III}) could be directly acylated with the formation of *acetyl-α-isostrophanthidic methyl ester*. Further studies were therefore made with this derivative. On catalytic reduction its aldehyde group was reduced with difficulty to the primary alcoholic group with the formation of *acetyl-α-isostrophanthidolic methyl ester*. Attempts to replace the primary hydroxyl group of this substance by halogen were unsuccessful. When it was treated with thionyl chloride, the acetyl group was replaced by thionyl with the formation of a neutral sulfite in which the primary and secondary hydroxyl groups were bridged by SO. In the course of these studies it was found that isostrophanthidic methyl ester also forms a neutral sulfite when treated with thionyl chloride; but in this case the SO group bridged the tertiary hydroxyl (OH^{II}) and the secondary hydroxyl (OH^{III}).

[4] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 831 (1927).

[5] Reduction to the primary alcohol was also accomplished by the use of sodium amalgam after saponification of the lactone group of α-isostrophanthidic acid and neutralization with acetic acid and addition of $(NH_4)_2SO_4$ as a buffer.

EXPERIMENTAL

Semicarbazone of α-Isostrophanthidic Acid—To a solution of 10 gm. of α-isostrophanthidic acid in 800 cc. of alcohol were added solutions of 10 gm. of potassium acetate in 55 cc. of alcohol and 10 gm. of semicarbazide hydrochloride in 130 cc. of water. The mixture was allowed to stand 48 hours at 38–40°. It was then concentrated under reduced pressure until practically dry. The residue was warmed with a little water for removal of salts, and then collected with water. After recrystallization from dilute alcohol, it melts at 305° after preliminary darkening. The yield was 9 gm.

3.662 mg. substance: 2.480 mg. H_2O, 8.105 mg. CO_2.
3.805 " " : 0.297 cc. N_2 (24.5°, 760.2 mm.).

$C_{24}H_{35}O_7N_3$.	Calculated.	C 60.34,	H 7.40,	N 8.80
	Found. (*a*)	" 60.36,	" 7.58	
	(*b*)			N 8.97

Desoxo-α-Isostrophanthidic Acid (*Isoperiplogenic Acid*)—2 gm. of α-isostrophanthidic acid semicarbazone were placed in a dry bomb tube with 50 cc. of absolute alcohol. To this were added 1.5 gm. of sodium. After solution of the sodium the tube was sealed and heated at 180° for 16 hours. The contents of the tube were washed out with water, and the solution was just acidified to Congo red and then allowed to stand in order to permit relactonization. At this stage a portion of the separating solid formed a resinous mass and was removed on a stirring rod and then dissolved in slightly diluted acetone. The solution was just acidified to Congo red and after standing an hour to insure complete lactonization was carefully diluted and the partly crystalline deposit was collected and joined with the main portion. It was then recrystallized by careful dilution of a methyl alcoholic solution. After repeated recrystallizations it melted at 215–217° with decomposition. The substance in ammoniacal solution did not decolorize permanganate. In sulfuric acid it formed a deep amber-colored solution, changing to red with an olive reflex on standing. The substance crystallized with approximately 1 mol of water of crystallization which was removed completely only on drying at 110° and 15 mm.

$[\alpha]_D^{24} = -23.3°$ (c = 1.056 in alcohol).

4.803 mg. substance: 3.490 mg. H_2O, 11.905 mg. CO_2.

$C_{23}H_{34}O_6$. Calculated.	C 67.94,	H 8.45
Found.	" 67.60,	" 8.13

14.760 mg. of anhydrous substance were treated with 1 cc. of alcohol and titrated directly against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent for $C_{23}H_{34}O_6$, 0.363 cc. Found, 0.352 cc.

3 cc. of 0.1 N NaOH were then added and the solution was refluxed for 4 hours and then titrated back. Calculated for 1 equivalent, 0.363 cc. Found, 0.375 cc.

α-Isostrophanthidolic Methyl Ester—A solution of 1 gm. of α-isostrophanthidic acid in 65 cc. of methyl alcohol together with 20 cc. of HCl (1.19) was turbined with 25 gm. of amalgamated granulated zinc (30 mesh), during which operation the temperature was kept below 30°. After 1.5 hours the cooled solution was decanted and then most of the free acid was neutralized with alkali. The concentrated solution was then extracted with chloroform. The latter was washed with sodium carbonate solution. The residue obtained on evaporation of the solvent crystallized under methyl alcohol. On recrystallization from methyl alcohol the substance separated as leaflets which melted at 223°.

$[\alpha]_D^{24} = -18.5°$ (c = 1.24 in pyridine).

4.992 mg. substance: 3.693 mg. H_2O, 12.126 mg. CO_2.

4.653 " " : 3.410 " " 11.260 " "

$C_{24}H_{36}O_7$. Calculated.		C 66.01,	H 8.32
Found.	(a)	" 66.24,	" 8.28
	(b)	" 65.99,	" 8.20

For comparison the rotations of α-isostrophanthidic acid and its methyl ester were also taken in pyridine and gave respectively $[\alpha]_D^{20} = -14°$ (c = 1.03 in pyridine) and $[\alpha]_D^{20} = -15°$ (c = 1.01 in pyridine).

γ-Isostrophanthidolic Methyl Ester—1 gm. of α-isostrophanthidic acid dissolved in a mixture of 100 cc. of methyl alcohol and 15 cc. of HCl (1.19) was refluxed with 25 gm. of amalgamated zinc. After 40 minutes a further 15 cc. of acid were added and the mixture was heated for 35 minutes more. The reaction product was isolated as in the previous instance, but the yield of crystalline product was considerably less. The chloroform residue only

partly crystallized under methyl alcohol. After recrystallization from this solvent, 0.17 gm. of sparingly soluble needles was obtained, which melted at 229–231°.

$[\alpha]_D^{22} = +98°$ (c = 1.000 in pyridine).
4.315 mg. substance: 3.225 mg. H_2O, 10.480 mg. CO_2.
4.105 " " : 2.260 " AgI.
$C_{24}H_{36}O_7$. Calculated. C 66.01, H 8.32, OCH_3 7.14
Found. " 66.24, " 8.35
OCH_3 7.27

Acetyl Isostrophanthidic Methyl Ester.—2.5 gm. of isostrophanthidic methyl ester were covered in a distilling flask with 50 cc. of acetyl chloride. After a few minutes a rather vigorous reaction occurred with complete solution. The excess of reagent was removed under diminished pressure, and the residue was dissolved in methyl alcohol. The acetate deposited as prisms on careful dilution. It was further recrystallized from ethyl alcohol, and melted with decomposition at 156–157°. The substance possesses a variable melting point. Different preparations melted between 127° and 157°.

$[\alpha]_D^{22} = -16°$ (c = 0.907 in pyridine).
4.040 mg. substance: 2.760 mg. H_2O, 9.680 mg. CO_2.
$C_{26}H_{36}O_8$. Calculated. C 65.51, H 7.62
Found. " 65.35, " 7.64

12.510 mg. of substance were covered with 1 cc. of alcohol and 3 cc. of 0.1 N NaOH. The mixture was refluxed for $4\frac{1}{2}$ hours and was then titrated back against phenolphthalein. Calculated for 3 equivalents, 0.788 cc. Found, 0.810 cc.

Acetyl-α-Isostrophanthidolic Methyl Ester—1.0 gm. of the acetate of isostrophanthidic methyl ester was hydrogenated in ethyl alcohol with 0.2 gm. of platinum oxide catalyst. A slow, steady absorption of 1 mol of hydrogen occurred, which required about 4 days for completion. The filtered solution was concentrated to dryness. The methyl alcoholic solution on careful dilution deposited cubes which sintered at 125–126° and melted with decomposition at 145°.

$[\alpha]_D^{22} = -25°$ (c = 1.22 in pyridine).
3.740 mg. substance: 2.645 mg. H_2O, 8.720 mg. CO_2.
$C_{26}H_{38}O_8$. Calculated. C 65.23, H 8.00
Found. " 64.97, " 7.98

Sulfite of Isostrophanthidolic Methyl Ester—When the attempt was made to substitute the hydroxyl group of the above acetate with chlorine by means of thionyl chloride, a halogen-free substance was obtained, which proved to be the neutral sulfite. 0.1 gm. of the acetate was treated with 2 cc. of $SOCl_2$ at 0°. Immediate solution with effervescence occurred. After 45 minutes the excess reagent was removed under diminished pressure. The sulfite formed sparingly soluble crystals from methyl alcohol and melted at 220°.

4.557 mg. substance: 2.860 mg. H_2O, 9.968 mg. CO_2.
$C_{24}H_{34}O_8S$. Calculated. C 59.71, H 7.11
Found. " 59.66, " 7.02

Sulfite of α-Isostrophanthidic Methyl Ester—0.2 gm. of α-isostrophanthidic methyl ester was dissolved in 4 cc. of $SOCl_2$ at 0°. After removal of the excess reagent the ester became crystalline under acetone. When recrystallized from methyl alcohol it formed needles which melted at 228°.

$[\alpha]_D^{24} = -40°$ (c = 0.56 in pyridine).
3.829 mg. substance: 2.302 mg. H_2O, 8.374 mg. CO_2.
$C_{24}H_{32}O_8S$. Calculated. C 59.96, H 6.71
Found. " 59.64, " 6.72

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STROPHANTHIN

XXI. THE CORRELATION OF STROPHANTHIDIN AND PERIPLOGENIN

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(Received for publication, March 9, 1931)

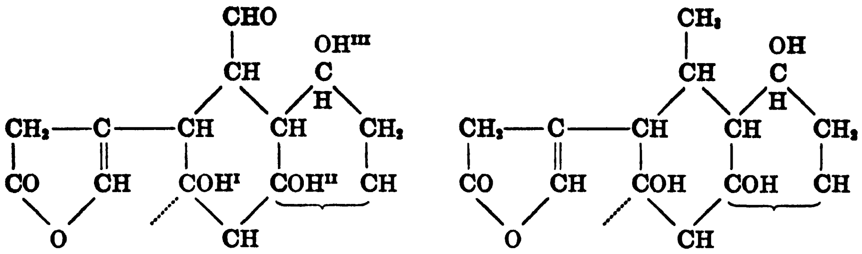
In a former communication,[1] the results were presented of an investigation on the cardiac glucosides which occur in *Periploca græca*. It was shown that by enzymatic cleavage of the glucosides present in a purified extract of this plant it is possible to obtain a chloroform-soluble substance, periplocymarin, which is a glucoside of the aglucone, periplogenin, with a methyl ether desoxy sugar, $C_7H_{14}O_4$. Periplogenin was found to possess the formula $C_{23}H_{34}O_5$ and to be a trihydroxytetracyclic-$\Delta^{\beta,\gamma}$-lactone. These facts at once suggested the probability of a close structural relationship with strophanthidin. This has now been substantiated by our more recent investigations.

In the preceding paper[2] the conversion of α-isostrophanthidic acid into its desoxo derivative has been described. This acid, $C_{23}H_{34}O_6$, has now proved on direct comparison to be identical with our previously described isoperiplogenic acid. This conclusion is supported by the results of a further comparison of the *methyl esters* of the acids obtained from both sources and of their oxidation products, *α-isoperiplogonic methyl ester* (*desoxo-α-isostrophanthidonic methyl ester*).

Isoperiplogenic acid is, therefore, desoxoisostrophanthidic acid and the same relationship can be directly assumed to hold for the parent aglucones. Periplogenin is, therefore, desoxostrophanthidin in which the aldehyde group of strophanthidin is replaced by methyl. Their partial formulæ may be given as follows:

[1] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 519 (1928).

[2] Jacobs, W. A., Elderfield, R. C., Grave, T. B., and Wignall, E. W., *J. Biol. Chem.*, **91**, 617 (1931).



Strophanthidin Periplogenin

We have in the meantime been able to procure more periplocymarin for an investigation of its sugar. This has been made possible through the kind cooperation of Doctor E. S. London of Leningrad, as well as Professor S. T. Petcheff of Suchum, Caucasus, and Doctor W. W. Stein of Sotshi, Caucasus, who arranged for the collection of a generous quantity of the plant *Periploca græca*. The sugar was found to be identical with cymarose, the methyl ether desoxy sugar which occurs in cymarin. Periplocymarin is therefore desoxocymarin.

EXPERIMENTAL

α-Isoperiplogenic Acid (Desoxo-α-Isostrophanthidic Acid)—In the previous report of our analytical experience with this acid prepared from periplogenin the results given were obtained after drying at 100° and 20 mm. and indicated the retention of 0.5 mol of water in the dried samples. Following the results obtained with desoxoisostrophanthidic acid as given in the preceding paper, isoperiplogenic acid was similarly dried at 110° and 20 mm. and now gave satisfactory results.

4.437 mg. substance: 3.270 mg. H_2O, 11.005 mg. CO_2.

$C_{23}H_{34}O_6$. Calculated.	C 67.94,	H 8.44
Found.	" 67.64,	" 8.25

The acids from both sources melted at 215–217° with decomposition and a mixture of the two showed no depression. The crystalline form, six-sided or diamond-shaped leaflets, was identical in both cases, as were other physical properties.

α-Isoperiplogenic Methyl Ester (Desoxo-α-Isostrophanthidic

Methyl Ester)—The methyl esters prepared from the acids by means of diazomethane in acetone solution were found to be identical. In each case recrystallization from acetone gave long prisms which melted at 242° (not 252° as previously reported for the periplogenin derivative). The mixture showed no depression. For the strophanthidin derivative $[\alpha]_D^{23} = -30.2°$ (c = 1.110 in pyridine). For the periplogenin derivative $[\alpha]_D = -33.2°$ (c = 0.965 in pyridine).

The analysis of the ester from periplogenin has already been recorded. That obtained with the strophanthidin derivative is as follows:

4.200 mg. substance: 3.255 mg. H_2O, 10.580 mg. CO_2.
5.783 " " : 3.338 " AgI.

$C_{24}H_{36}O_6$. Calculated.	C 68.51, H 8.64,	OCH_3 7.38
Found.	" 68.70, " 8.67	
		OCH_3 7.62

16.593 mg. of the ester of strophanthidin origin were refluxed for 4.5 hours in a mixture of 1 cc. of alcohol and 3 cc. of 0.1 N NaOH and then titrated back against phenolphthalein. Calculated for 2 equivalents, 0.788 cc. Found, 0.776 cc.

α-Isoperiplogonic Methyl Ester (*Desoxo-α-Isostrophanthidonic Methyl Ester*)—A solution of 0.5 gm. of desoxoisostrophanthidic methyl ester in 15 cc. of acetic acid was treated with 2.5 cc. of Kiliani's CrO_3 solution. After 10 minutes, dilution with water gave a crystalline precipitate. Recrystallized from methyl alcohol, the keto ester separated as sparingly soluble needles which melted at 228°.

$[\alpha]_D^{24} = -23°$ (c = 0.825 in pyridine).
3.905 mg. substance: 2.778 mg. H_2O, 9.872 mg. CO_2.

$C_{24}H_{34}O_6$. Calculated.	C 68.85, H 8.20
Found.	" 68.95, " 7.96

The identical keto ester was similarly prepared from isoperiplogenic methyl ester. It melted at 230° and showed no depression when mixed with the above oxidation product.

$[\alpha]_D = -23.2°$ (c = 1.145 in pyridine).
4.245 mg. substance: 3.020 mg. H_2O, 10.742 mg. CO_2.
Found. C 69.01, H 7.96

Cymarose from Periplocymarin—3 gm. of periplocymarin were shaken with a mixture of 21 cc. of alcohol, 13.5 cc. of water, and 6.7 cc. of HCl (1.19) until dissolved. The solution was left then at 20° for 3 hours. On dilution periplogenin separated. This was completed by allowing the mixture to stand at 0°. The filtrate was neutralized with an excess of Ag_2CO_3 and the Cl-free solution was treated with H_2S. The sugar solution after concentration to about 50 cc. was extracted with chloroform to remove impurities. The concentration to dryness was then completed. The residue was extracted with anhydrous ether and the ether solution of the sugar after partial concentration was diluted with petrolic ether. On seeding with cymarose the sugar separated slowly as beautiful long needles. After recrystallization from ether-petrolic ether the sugar melted at 100–102°, after considerable preliminary softening. This melting point was obtained, however, only after drying for several days over $CaCl_2$. Short exposure to moist air at once depressed the melting point. This explains the lower melting point (93°) previously recorded.[3] No depression in melting point was found on mixing the cymaroses from both sources.

$[\alpha]_D^{20} = +52°$ (c = 1.000 in water).
3.696 mg. substance: 2.860 mg. H_2O, 7.042 mg. CO_2.
3.862 " " : 5.565 " AgI.
$C_7H_{14}O_4$. Calculated. C 51.81, H 8.70, OCH_3 19.13
Found. " 51.96, " 8.66
OCH_3 19.04

Addendum—In more recent work digitoxigenin and therefore gitoxigenin have been correlated with strophanthidin and periplogenin. Isoperiplogonic methyl ester which has just been described in these pages on dehydration loses OH^{II} and the resulting *anhydroisoperiplogonic methyl ester* on hydrogenation yields a mixture of isomeric desoxy derivatives. One of these has been found to be identical with isodigitoxigonic methyl ester in melting point, rotation, and crystalline form. This was confirmed by a further comparison of the desoxyisoperiplogonic acid obtained on saponification of the ester with isodigitoxigonic acid. Digitoxigenin is therefore desoxyperiplogenin and desoxodesoxystrophanthidin. The details of this work will soon follow.

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[3] Jacobs, W. A., *J. Biol. Chem.*, 88, 527 (1930).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, July, 1931,
Vol. 92, No. 2, pp. 313–321]

STROPHANTHIN

XXII. THE CORRELATION OF STROPHANTHIDIN AND PERIPLOGENIN WITH DIGITOXIGENIN AND GITOXIGENIN

BY WALTER A. JACOBS AND ROBERT C. ELDERFIELD

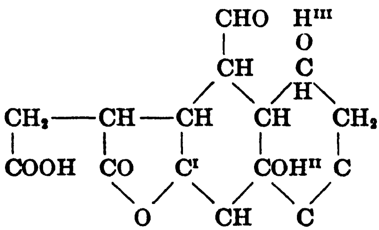
(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, May 23, 1931)

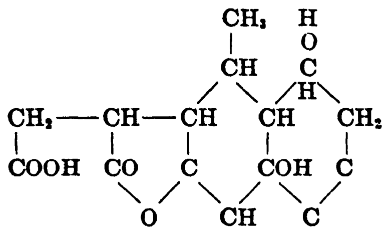
In a recent communication[1] the structural relationship between strophanthidin and periplogenin has been described. This correlation was made possible by the conversion of α-isostrophanthidic acid (partial formula I) into its desoxo derivative (II), which was in turn found to be identical with isoperiplogenic acid. This identity was confirmed by a comparison of the methyl esters obtained from both sources as well as by the comparison of the oxidation products of these esters in which the secondary hydroxyl group has been oxidized to the ketone, isoperiplogonic methyl ester (IV). Our experience therefore in regard to transformations with certain derivatives of isostrophanthidin at once gave promise of becoming applicable to analogous derivatives of isoperiplogenin. α-Isostrophanthonic dimethyl ester (III) differs from isoperiplogonic methyl ester (IV) by its possession of a carbomethoxyl group in place of the methyl group of the latter compound. In previous work[2] it has been shown that since OH^{II} is in a position β to the carbonyl group, isostrophanthonic dimethyl ester may be readily converted into the unsaturated anhydroisostrophanthonic dimethyl ester. Isoperiplogonic methyl ester has now been found to yield very readily the analogous unsaturated *anhydroisoperiplogonic methyl ester* (V). And just as the former unsaturated compound on hydrogenation with palladium and hydrogen was converted into a mixture of stereoisomeric saturated desoxy-α-

[1] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **91**, 625 (1931).
[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 813 (1927).

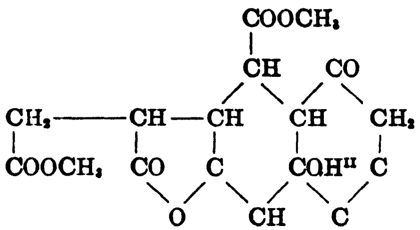
isostrophanthonic dimethyl esters, anhydroisoperiplogonic methyl ester was found to behave similarly. The latter on hydrogenation yielded a mixture of saturated *desoxyisoperiplogonic methyl esters* (VI) from which two isomers have been isolated. One of these was more readily separated because of its sparing solubility. It melted at 251°. The second isomer which appeared to have been formed in smaller amount was separated from other more soluble hydrogenation products. After repeated recrystallization from ether it was obtained in a pure form which melted at 192–193.5°.



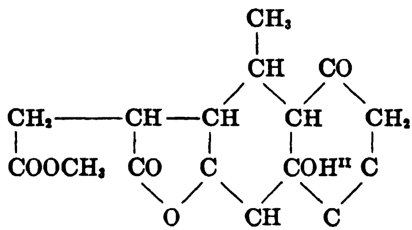
I



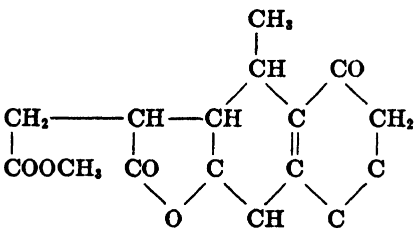
II



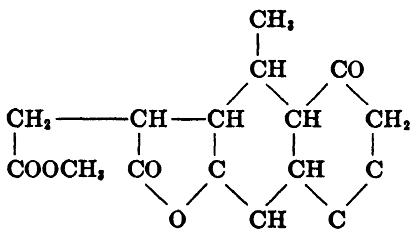
III



IV



V



VI

In previous work on digitoxigenin reported from this laboratory[3] its conversion into isodigitoxigenin was described and the rela-

[3] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **78**, 573 (1928).

tionship of this iso derivative to its parent aglucone was shown to be the same as that which had already been established between isostrophanthidin and strophanthidin. In the course of this work isodigitoxigenin on saponification of its lactone group was converted into the lactol acid, isodigitoxigeninic acid. The latter as the methyl ester was oxidized by chromic acid to the lactone ester, isodigitoxigonic methyl ester, in which the secondary acylatable hydroxyl group was simultaneously oxidized to carbonyl. This keto lactone ester has the same formula, $C_{23}H_{34}O_5$, as the above desoxyisoperiplogonic methyl ester obtained from periplogenin. Since the melting point of the digitoxigenin derivative was practically the same as that observed in the case of the more soluble of the two desoxyisoperiplogonic methyl esters it was of interest to make a careful direct comparison of the substances obtained from both sources. We believe that this comparison has definitely established the identity of the isodigitoxigonic methyl ester and the more soluble desoxyisoperiplogonic methyl ester. This was shown by melting points, mixed melting points, crystalline form, rotation, and general properties. Further, on saponification, the periplogenin derivative yielded a *desoxyisoperiplogonic acid* which could not be distinguished from isodigitoxigonic acid, as regards melting point, crystalline form, and other properties.

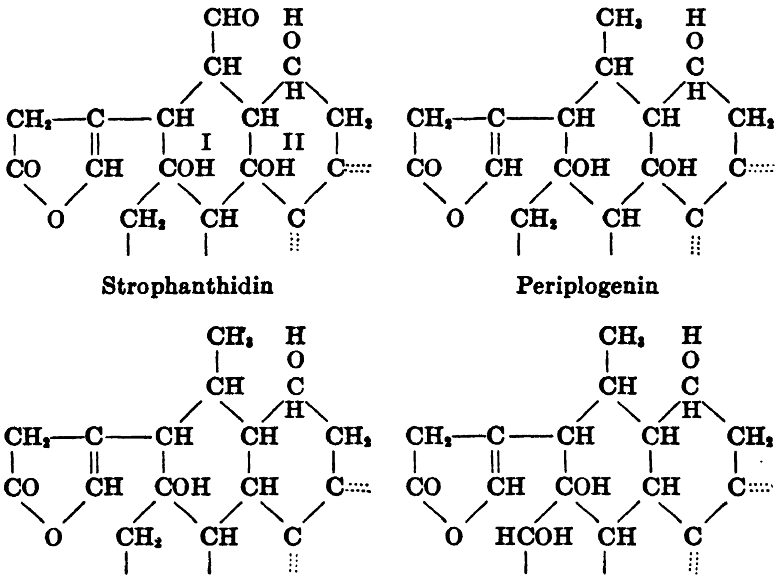
For additional confirmation of the identity of the substances from both sources a comparison was made of the substances obtained from each by isomerization with hydrochloric acid. Isodigitoxigonic acid was converted by this reagent into *γ-isodigitoxigonic acid* which was characterized further by its *methyl ester*. Desoxyisoperiplogonic acid of strophanthidin origin yielded similarly a *γ-acid* and *methyl ester* which agreed in properties with the above *γ-derivatives* from digitoxigenin.

From these results periplogenin is hydroxydigitoxigenin in which the extra hydroxyl group is OH^{II} of the strophanthidin and periplogenin molecules. The correlation of strophanthidin, periplogenin, and digitoxigenin appears, therefore, to be definitely established. Since gitoxigenin has been shown to be hydroxydigitoxigenin,[4] the isomerism of gitoxigenin and periplogenin both of the formula, $C_{23}H_{34}O_5$, consists solely in the position of this extra

[4] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 199 (1930).

hydroxyl. In periplogenin it is of tertiary and in gitoxigenin of secondary nature.

In the work of Windaus, Westphal, and Stein[5] hexahydrodigitaligenin has been shown to yield a ketone which on further oxidation gives rise to a dibasic acid with ring cleavage. On distillation this acid gives a "Brenzketon," thus establishing the fact that the secondary hydroxyl group is attached to a carbon atom of a 6- or possibly a 7-membered ring. The results will be published shortly of a study of ring II of strophanthidin, which definitely restrict its size to a 6-membered ring. The relationships between the four aglucones—strophanthidin, periplogenin, digitoxigenin, and gitoxigenin—are therefore given in the following partial formulæ.



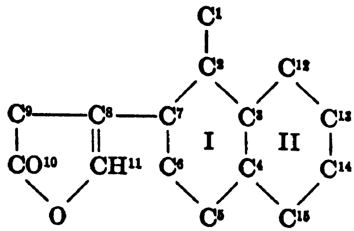
Strophanthidin Periplogenin

Digitoxigenin Gitoxigenin

In these formulæ the unsaturated lactone side chain is for the moment arbitrarily attached to carbon atom (7) although carbon atom (5) is also a possibility. This question is at the moment under investigation. The numerical designations given to the

[5] Windaus, A., Westphal, K., and Stein, G., *Ber. chem. Ges.*, **61**, 1854 (1928).

carbon atoms of the known portion of the molecule are as follows:



The structural differences between these four aglucones, with the exception of the aldehyde group of strophanthidin, are thus seen to be a function merely of the number and position of the hydroxyl groups. The same situation will without doubt be found in the case of the recently isolated *Digitalis lanata* aglucone, digoxigenin ($C_{23}H_{34}O_5$),[6] and perhaps in the case of sarmentogenin,[7] of the aglucone of ouabain, of antiarigenin (this possesses also a CO group), and of other aglucones of this group of substances. It will probably be found that in the naturally occurring glucosides of these aglucones the point of glucosidic union of the sugar on the aglucone is on the secondary acylatable hydroxyl group. This has already been established in the case of cymarin.[8]

Although the general pharmacodynamic action of the cardiac glucosides is roughly a common one, it is interesting that not only possible differences in the character of their actions but also marked differences in the intensity of their effects have been noted. It is possible that not only the number and position of the substituting hydroxyl groups of the aglucones determine such quantitative and qualitative differences but it is also possible that the number and nature of the sugar components of the individual glucosides may play a rôle in determining such differences in action.

We are indebted to E. Merck, Darmstadt, for the "insoluble digitoxin by-product" which was the source of the digitoxigenin employed in these studies.

[6] Smith, S., *J. Chem. Soc.*, **509**, 2478 (1930). Mannich, C., Mohns, P., and Mauss, W., *Arch. Pharm.*, **268**, 453 (1930).

[7] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, **81**, 765 (1929).

[8] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **67**, 614 (1926).

EXPERIMENTAL

Anhydroisoperiplogonic Methyl Ester—0.4 gm. of isoperiplogonic methyl ester (of both strophanthidin and periplogenin origin) was refluxed for 18 minutes in a mixture of 30 cc. of methyl alcohol and 1.2 cc. of hydrochloric acid (1.19). The solution was diluted with water and a moderate excess of sodium acetate solution was added. The lustrous platelets which separated were collected with water. The yield was 0.35 gm. After recrystallization from methyl alcohol, the keto ester formed sparingly soluble broad needles or flat prisms which melted at 225–226° after preliminary softening.

3.962 mg. substance: 2.942 mg. H_2O, 10.430 mg. CO_2.

$C_{24}H_{32}O_5$.	Calculated.	C 71.94, H 8.07
	Found.	" 71.80, " 8.30

Desoxyisoperiplogonic Methyl Ester (Isodigitoxigonic Methyl Ester)—0.6 gm. of anhydroisoperiplogonic methyl ester in acetic acid solution was shaken with 0.1 gm. of palladium black and hydrogen. 1 mol of H_2 was absorbed during 30 minutes. The filtered solution was concentrated to dryness under diminished pressure and the residue was dissolved in methyl alcohol. Concentration of the resulting solution yielded three crops of a rather sparingly soluble substance. When recrystallized from methyl alcohol it separated as long flat needles or plates melting at 251–252°. 0.18 gm. of pure substance was thus obtained.

$[\alpha]_D^{24} = -44°$ (c = 1.250 in pyridine).
3.580 mg. substance: 2.820 mg. H_2O, 9.417 mg. CO_2.

$C_{24}H_{34}O_5$.	Calculated.	C 71.60, H 8.52
	Found.	" 71.74, " 8.80

The mother liquor from the final crop of the above substance amounted to about 5 cc. On dilution with water, a voluminous precipitate formed which was collected with water. After repeated recrystallization from ether, 0.15 gm. was obtained as rectangular platelets which melted at 192–193.5°. An identical melting point was shown by isodigitoxigonic methyl ester which had been obtained from digitoxigenin. A mixture of the two substances showed no depression. The substances from both sources were again recrystallized from ether and their melting points as well as that of the mixture remained unchanged. Both substances

gave a practically colorless solution in H_2SO_4, which very slowly changed on warming to a pale yellow with slight greenish fluorescence.

The substances of periplogenin and digitoxigenin origins gave the following rotations respectively.

$[\alpha]_D^{25} = -40.5°$ (c = 1.025 in alcohol).
$[\alpha]_D^{25} = -39.5°$ (c = 0.905 in alcohol).

The analysis of the substance from digitoxigenin has already been reported.[3] The analytical figures obtained from the periplogenin product are as follows:

4.057 mg. substance: 3.165 mg. H_2O, 10.680 mg. CO_2.

$C_{24}H_{34}O_5$.	Calculated.	C 71.60, H 8.52
	Found.	" 71.79, " 8.73

The same series of substances was obtained from isoperiplogonic methyl ester of strophanthidin origin.

Desoxyisoperiplogonic Acid (Isodigitoxigonic Acid)—The more soluble desoxyisoperiplogonic methyl ester was saponified by gentle warming of a suspension in dilute alcoholic solution containing a slight excess of alkali. On gentle acidification the acid separated as flat needles. After collection with water it was recrystallized by careful dilution of an alcoholic solution. It deposited as flat needles or platelets which were indistinguishable in form from isodigitoxigonic acid. It melted at 206–208° and showed no depression when mixed with a freshly recrystallized sample of isodigitoxigonic acid. The latter melted identically and not at 212–213° as previously reported.[3] The analysis of the material from digitoxigenin has been reported; that for the periplogenin derivative was as follows:

3.910 mg. substance: 2.905 mg. H_2O, 10.220 mg. CO_2.

$C_{23}H_{32}O_5$.	Calculated.	C 71.08, H 8.31
	Found.	" 71.28, " 8.31

γ-Isodigitoxigonic Acid (γ-Desoxyisoperiplogonic Acid)—50 mg. of isodigitoxigonic acid were treated with 1 cc. of HCl (1.19) at 20°. Solution occurred readily and after a few minutes crystals of the γ-acid appeared. After standing 45 minutes the solution was carefully diluted and the crystals were collected with water.

The substance was recrystallized by careful dilution of its acetone solution from which it separated as long, hexagonal platelets which melted at 225-226°.

$[\alpha]_D^{25} = +70° \pm 2°$ (c = 0.555 in methyl alcohol).

For analysis the substance was dried at 100° and 15 mm.

4.265 mg. substance: 3.200 mg. H_2O, 11.125 mg. CO_2.
$C_{23}H_{32}O_5$. Calculated. C 71.08, H 8.31
Found. " 71.14, " 8.39

For comparison the same procedure was used to isomerize desoxyisoperiplogonic acid. The substance obtained was indistinguishable in form from the above acid. It melted at 225–226° and showed no depression when mixed with the above acid.

$[\alpha]_D^{25} = +67° \pm 2°$ (c = 0.535 in methyl alcohol).
4.294 mg. substance: 3.145 mg. H_2O, 11.235 mg. CO_2.
$C_{23}H_{32}O_5$. Calculated. C 71.08, H 8.31
Found. " 71.36, " 8.20

γ-Isodigitoxigonic Methyl Ester (γ-Desoxyisoperiplogonic Methyl Ester)—γ-Isodigitoxigonic acid was esterified in acetone solution with diazomethane. The ester was recrystallized by addition of dry ether to the concentrated acetone solution. It crystallized as lustrous, mostly hexagonal platelets, and melted at 179.5–180.5° after slight preliminary sintering. For analysis it was dried at 105° and 15 mm.

4.450 mg. substance: 3.447 mg. H_2O, 11.688 mg. CO_2.
$C_{24}H_{34}O_5$. Calculated. C 71.60, H 8.52
Found. " 71.64 " 8.67

For comparison the same procedure was used to esterify γ-desoxyisoperiplogonic acid. This ester was identical with the above ester in crystalline form and general properties. It melted at 180–181° with slight preliminary sintering and showed no depression when mixed with the above ester.

4.192 mg. substance: 3.273 mg. H_2O, 11.025 mg. CO_2.
Found. C 71.73, H. 8.74

The same substance was also prepared by oxidation of γ-isodigitoxigenic methyl ester in acetic acid solution with Kiliani's CrO_3 solution.

Isoperiplogenic Acid from α-Isostrophanthidic Acid Semicarbazone—In more recent work it has been found desirable to modify somewhat the procedure previously given for the preparation of desoxoisostrophanthidic acid. The contents of the bomb tubes were washed into a separatory funnel with water, the solution was made acid to Congo red with hydrochloric acid and then immediately extracted with chloroform before crystallization occurred. The resin remaining after concentration of the chloroform solution was dissolved in acetone. The solution was diluted slightly and then acidified to Congo red. On standing, crystallization occurred which was facilitated by further dilution. The substance was then recrystallized by careful dilution of its acetone solution.

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STROPHANTHIN

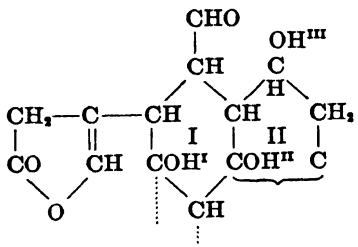
XXIII. RING II OF STROPHANTHIDIN AND OF RELATED AGLUCONES

BY WALTER A. JACOBS AND EDWIN L. GUSTUS

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, May 21, 1931)

In the strophanthidin molecule one of its four rings which has been designated as Ring I (Formula I) has been found to be 6-membered. This conclusion has been based upon a number of previously presented observations, especially those which have had to do with the behavior of trianhydrostrophanthidin on hydrogenation and on oxidation.[1] Adjoining Ring I is a second ring, Ring II,



I

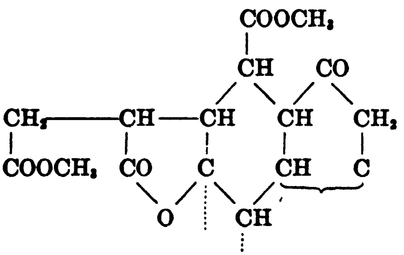
to which is attached the secondary, acylatable hydroxyl group (OH^{III}) and the determination of the size of this ring has been the subject of long inquiry by us. This we believe has now been brought to a definite conclusion.

The solution of the problem was first attempted by the application of the usual procedure of oxidizing the secondary hydroxyl group (OH^{III}) to carbonyl and then attempting ring cleavage to a dicarbonic acid which on distillation should yield either an anhydride or a cyclic ketone. Unfortunately this method was not

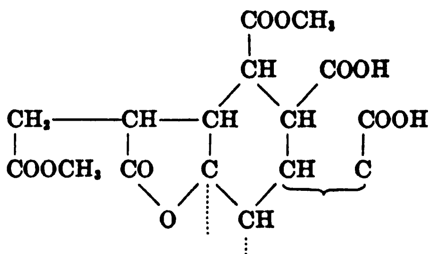
[1] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 805 (1927).

applicable to strophanthidin itself or its immediate derivatives because of complications caused by the proximity of the tertiary hydroxyl (OH^{II}) which is β to any resulting ketone. In addition, the secondary hydroxyl^{III} (and therefore the ketonic group) is γ to the aldehyde group (or carboxyl group produced from it) attached to Ring I.

In order to circumvent these difficulties attempts were made to accomplish this purpose with the two previously described[2] hydrogenation products of anhydroisostrophanthonic dimethyl ester, the isomeric desoxy-α-isostrophanthonic dimethyl esters (II) in which OH^{II} has been replaced by hydrogen and the carboxyl group has been converted into a relatively stable ester group. One of these isomers ($[\alpha] = +12$), which was formed in larger amount during hydrogenation, gave on oxidation only non-crystalline products which proved to be useless for our purpose. The isomer obtainable in smaller amount yielded, however, a crystalline dibasic acid, $C_{25}H_{34}O_{10}$ (III).



II



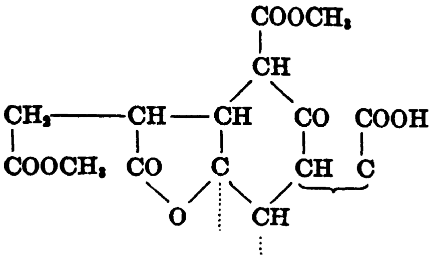
III

This dibasic acid on heating with acetic anhydride was easily converted into a crystalline anhydride. On attempting, however, thermic decomposition of the latter, no crystalline reaction product could be obtained. Apparently the stable carbomethoxyl group was involved in the decomposition, a fact which was indicated by analysis of the resulting resin for methoxyl. Fortunately, however, another method of approach became available.

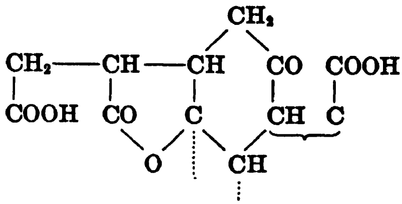
It has already been shown[3] that anhydroisosthrophanthonic dimethyl ester on oxidation is degraded with the loss of its ketonic

[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 74, 820 (1927).
[3] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 79, 539 (1928).

carbon atom and the formation of a keto acid (IV), undephanthontriacid dimethyl ester.



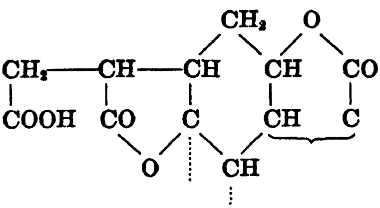
IV



V

This acid was shown to be a β-ketonic ester which readily undergoes the ketonic decomposition to form the keto acid, duodephanthondiacid (V).

In more recent work which will be described in the experimental part, the latter and even its dimethyl ester on reduction with catalytically activated hydrogen have given at once lactones (VI) due to the prompt lactonization of the free carboxyl group on the hydroxyl group produced by reduction of the carbonyl group. This ready lactonization practically restricts duodephanthondiacid



VI

within the category of a γ- or δ-keto acid. Ring II of strophanthidin therefore must be either a 5- or a 6-membered ring. The decision between the two possibilities appeared to rest on the determination of whether duodephanthondiacid is a γ- or a δ-ketonic acid.

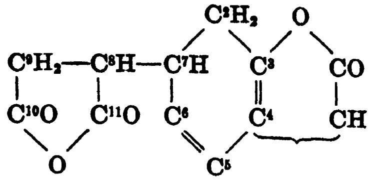
A possible method which was suggested for deciding this point was a study of the behavior of this acid on lactonization. γ-Ketonic acids on lactonization yield $\Delta^{\beta,\gamma}$-unsaturated lactones while in the case of a δ-ketonic acid the product is a $\Delta^{\gamma,\delta}$-un-

saturated lactone. It has been our experience that $\Delta^{\beta,\gamma}$-unsaturated lactones which possess a free hydrogen in the α-position give the nitroprusside reaction.[4] It was hoped, therefore, that the lactone obtained from duodephanthondiacid, when investigated from this standpoint, would yield the desired evidence. A positive Legal reaction would indicate a $\Delta^{\beta,\gamma}$-lactone and therefore a γ-keto acid, while a negative reaction would indicate a $\Delta^{\gamma,\delta}$-lactone or possibly the unlikely case of a $\Delta^{\beta,\gamma}$-lactone with a quartenary α-carbon atom.

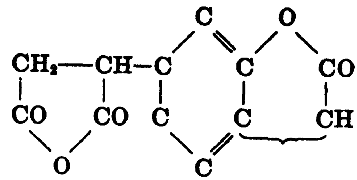
When an attempt was made to lactonize duodephanthondiacid directly by heating it above its melting point, only a non-crystalline reaction product was recovered. A crystalline substance, however, was readily obtained when the ketonic acid was heated with an acetic anhydride-acetyl chloride mixture. This substance proved to be of unexpected character and on analysis was found to have the formula $C_{21}H_{24}O_5$. The interpretation of the nature of this substance was aided by our previously reported study of the action of acetic anhydride-acetyl chloride on β-isostrophanthic lactone acid.[5] It was found that the reagent had not only accomplished the lactonization desired but had also attacked another portion of the molecule. This consisted in the cleavage of the original lactone group with the formation of a substituted succinic anhydride with simultaneous loss of OH^{I} to form an additional double bond. The resulting substance, therefore, was a doubly unsaturated lactone anhydride (VII). It was found to give no reaction with sodium nitroprusside. Although this result indicated that the substance is in all probability a $\Delta^{\gamma,\delta}$-lactone and that duodephanthondiacid is therefore a δ-ketonic acid, caution was required because of the complicating presence of the extra double bond. The latter might conceivably have induced a secondary shift of the double bond produced by lactonization on the keto group. If the probable assumption is made that the two double bonds are conjugated then six arrangements of these bonds in Ring I are possible as shown in Formulæ VII to XII.

[4] Jacobs, W. A., Hoffmann, A., and Gustus, E. L., *J. Biol. Chem.*, **70**, 1 (1926).

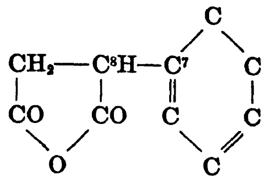
[5] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **84**, 183 (1929).



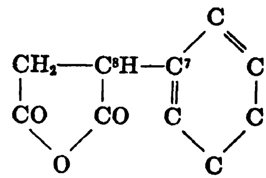
VII



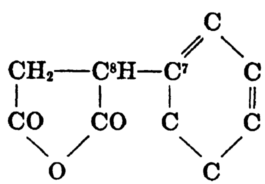
VIII



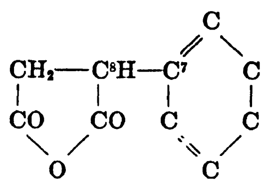
IX



X



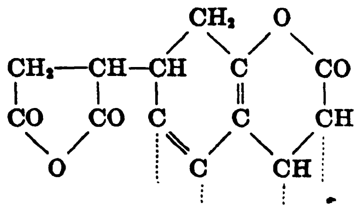
XI



XII

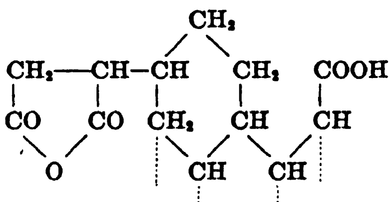
In Formulæ IX to XII a double bond is attached on one or the other side of carbon atom (7). In each of these cases there would therefore be a double bond β,γ to the proximal carbonyl of the anhydride group, and the intermediate carbon atom (8) would bear a hydrogen atom which should be active and give a positive Legal reaction. If such an assumption is acceptable, then these four arrangements are excluded from consideration because of the negative outcome of the Legal test with our substance. There remain, therefore, as possible arrangements free from this objection Formulæ VII and VIII, in which the other double bond is attached to carbon atom (3) which is the point of lactonization in question. If this were a γ-lactone then in both cases the α-carbon (if not quartenary) would bear an active hydrogen. Therefore, only if we are dealing with a $\Delta^{\gamma,\delta}$-unsaturated lactone could we properly expect a negative reaction. If this argument is permissible then Formula XIII, which contains the most probable

arrangement for the double bonds, appears to be the most acceptable partial formula for this unsaturated lactone anhydride.



XIII

That a double bond is still at the point of lactonization is supported by the behavior of this substance on catalytic hydrogenation. The hydrogenation was found to go beyond the stage of the saturation merely of the two double bonds. It absorbed readily 3 mols of hydrogen and the resulting product proved to be a mixture apparently of isomeric desoxy acids. One of these anhydride acids (XIV) was obtained in crystalline form and on analysis gave figures in agreement with the required formulation, $C_{21}H_{30}O_5$.



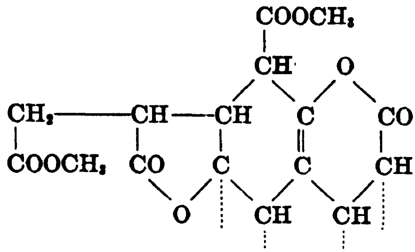
XIV

On saponification its anhydride group was readily opened with the formation of *the tribasic acid*, $C_{21}H_{32}O_6$, which was further characterized as *the trimethyl ester.*

If in the above lactonization of duodephanthondiacid the anhydride formation and consequent production of the additional double bond could have been prevented the evidence obtained and conclusions reached would have been based on fewer assumptions. This might have been accomplished by lactonization of the half-ester of this acid. The ester group would have prevented formation of the anhydride group with simultaneous production of the extra double bond. Unfortunately all attempts to realize this

plan by selective esterification of the one carboxyl group were unsuccessful.

When undephanthontriacid dimethyl ester was heated with acetic anhydride-acetyl chloride, it was readily converted into an *unsaturated lactone*, $C_{24}H_{30}O_8$ (XV), and because of the protecting ester group without the complication of anhydride formation and the production of a second double bond. The Legal test with this



XV

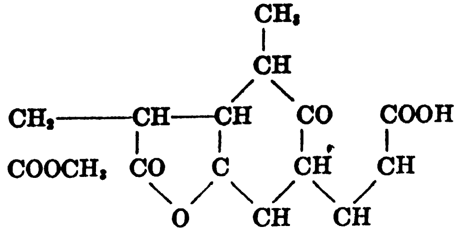
substance was strongly positive. Undephanthontriacid dimethyl ester (IV) itself, however, also gives a Legal test as a β-ketonic ester. In the case of its unsaturated lactone the test may be given by the same active H atom which lies between the ester CO group and the double bond β,γ to the latter. Such a test is therefore inconclusive in this case.

Fortunately, however, the recent correlation of periplogenin with strophanthidin[6] has made it possible to obtain the desired confirmatory evidence by another method of approach. For this purpose we have prepared the substance analogous to undephanthontriacid dimethyl ester from periplogenin. In a recent communication[7] a series of reactions has been described by which isoperiplogenic acid has been converted into anhydroisoperiplogonic methyl ester. This substance bears the same relationship to isoperiplogenin which anhydroisostrophanthonic dimethyl ester bears to isostrophanthidin. In the former ester a methyl group occupies the place of a carbomethoxyl group in the latter.

Anhydroisoperiplogonic methyl ester on oxidation with ozone yielded a degradation product which proved to be a ketonic acid,

[6] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **91**, 625 (1931).
[7] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **92**, 313 (1931).

$C_{22}H_{32}O_7$, with one less carbon atom, and has been designated *undeplogondiacid monomethyl ester* (XVI).



XVI

This ketonic acid, unlike the β-ketonic ester, undephanthontriacid dimethyl ester, gives no Legal reaction since the ester group of the latter which is β to the CO group is replaced by a methyl group.

Undeplogondiacid monomethyl ester is readily lactonized by the acetic anhydride-acetyl chloride method. The resulting *unsaturated lactone* gives with nitroprusside a faint test which we regard as definitely atypical and therefore practically negative. It is possible that the faint atypical color is attributable to some impurity or to a shift in the double bond under the influence of alkali during the test.

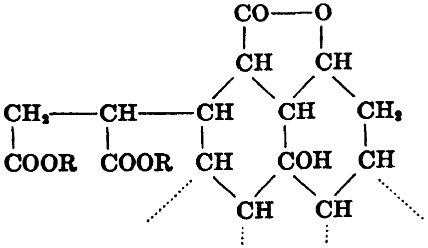
From these results all indications seem to point definitely to the conclusion that the ketonic acids which result from the degradation of anhydroisostrophanthonic dimethyl ester and anhydroisoperiplogonic methyl ester are δ-ketonic acids and that Ring II of both strophanthidin and periplogenin is therefore 6-membered.

This conclusion has recently received confirmation from another source. In previously reported work, Windaus, Westphal, and Stein[8] have succeeded in oxidizing the secondary hydroxyl group of hexahydrodigitaligenin through the ketone to the dibasic acid, $C_{23}H_{34}O_6$, with ring cleavage. This acid on distillation yielded a "Brenzketon," $C_{22}H_{32}O_3$. From this result they have properly concluded that the ring of gitoxigenin and digitaligenin which bears the secondary hydroxyl group is 6- or possibly 7-membered. In the recent work from our laboratory on the correlation of both

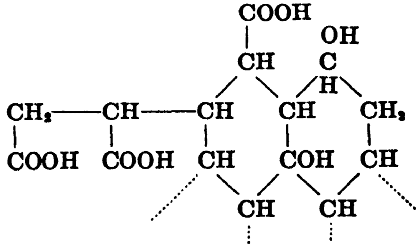
[8] Windaus, A., Westphal, K., and Stein, G., *Ber. chem. Ges.*, **61**, 1847 (1928). Stein, G., Inaugural dissertation, University of Göttingen (1929).

digitoxigenin and gitoxigenin with strophanthidin and periplogenin,[7] it has been established among other things that the secondary acylatable hydroxyl group is one of the features common to all of these aglucones. The results, therefore, of Windaus, Westphal, and Stein are at once applicable to the problem of the size of Ring II of strophanthidin. The only ring size which is compatible both with the formation of a pyroketone and with the ready lactonization of the above ketonic acids is a 6-membered ring. Ring II of the four cardiac aglucones must be, therefore, a substituted cyclohexane ring.

In the course of the work related to the problem of the size of Ring II several other methods of approach were attempted. Before the recent work leading to the preparation and study of undeplogondiacid monomethyl ester and its lactone, a plan had been formulated based on the use of β-isostrophanthic lactone acid.[9] The opening of the labile lactone group of this substance with loss of hydroxyl^{I} has already been described,[5] in which either acetic anhydride-acetyl chloride or methyl alcoholic hydrochloric acid was employed. Different stereoisomeric unsaturated dimethyl esters were obtained by the two methods. The ester obtained directly with methyl alcoholic hydrochloric acid, although unsaturated, resisted all efforts to hydrogenate its double bond catalytically. On the other hand the ester obtained through the intermediate anhydride was hydrogenated, although with difficulty, to the saturated desoxylactone dimethyl ester, $C_{25}H_{36}O_7$ (XVII). The stable lactone group of this substance involving carboxyl^{I} and hydroxyl^{III} was subsequently saponified with difficulty to the saturated *desoxytriacid*, $C_{23}H_{34}O_8$ (XVIII).



XVII

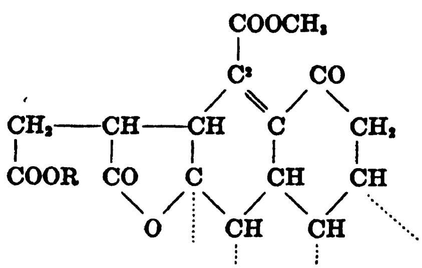


XVIII

[9] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 74, 829 (1927).

This acid was readily converted into the *trimethyl ester*. The secondary hydroxyl (OH^{III}) of this substance was easily oxidized to carbonyl with the formation of the *ketonic ester*. All further plans with this series of substances were frustrated at the next step when unsuccessful attempts were made to prepare the anhydro ketone by removal of hydroxyl^{II}. For this purpose the method was tried which has been repeatedly used with success for the preparation of the analogous anhydroisostrophanthonic dimethyl ester and anhydroisoperiplogonic methyl ester. This procedure as well as modifications have given only non-crystalline resins.

In the course of this work we have had occasion to prepare *β-isostrophanthonic dimethyl ester* from β-isostrophanthic dimethyl ester[9] with chromic acid. As expected, this substance readily lost water to form an *anhydroisostrophanthonic dimethyl ester*. Examination of this substance proved it to be identical with the anhydro ester already prepared from α-isostrophanthonic dimethyl ester. The identity of the anhydro ketones from both the α and β series confirms the position of the double bond already suggested for this substance.[8] Since the anhydro ketone gave no reaction with nitroprusside it had been suggested that the double bond formed by loss



XIX

of OH^{II} must shift to the position given in Formula XIX. Since the isomerism of α- and β-isostrophanthic acids and their derivatives has been shown to be due to the asymmetry of C^2 of the strophanthidin molecule, such asymmetry is lost by the double bond attached to it in anhydroisostrophanthonic dimethyl ester. The same anhydro ester should, therefore, be formed from the epimeric α- and β-isostrophanthonic esters.

EXPERIMENTAL

Part A

Undephanthontriacid Dimethyl Ester—A great improvement in the yield of this substance over that previously reported[8] in the oxidation of anhydroisostrophanthonic dimethyl ester was obtained by the use of ozone as the oxidizing agent. A stream of ozonized oxygen (about 1 to 1.5 per cent) was passed through a solution of 3 gm. of the dimethyl ester in 70 cc. of acetic acid kept at 20° at the rate of 1 liter per minute for 1 hour. The clear solution was concentrated under diminished pressure to small volume. After the addition of 300 cc. of water, the mixture was heated for a time on the water bath to decompose any ozonides. During this operation all with the exception of a small amount of oil dissolved. On being cooled, undephanthontriacid dimethyl ester gradually crystallized. The yield of crude material varied from 2 to 2.5 gm. Additional substance was recovered by extraction of the aqueous filtrate, and this was suitable for conversion into duodephanthondiacid. After recrystallization from methyl alcohol it formed leaflets which melted at 167–168°, somewhat lower than was observed with the substance previously reported (179–180°).

3.160 mg. substance: 1.963 mg. H_2O, 7.168 mg. CO_2.

$C_{24}H_{32}O_9$.	Calculated.	C 62.03, H 6.95
	Found.	" 61.86, " 6.95

In view of the discrepancy in melting points, the identity of the substances obtained by both methods was checked by a comparison of the same derivatives prepared from each. The trimethyl ester obtained by the action of diazomethane on the ozone product melted at 149–151° and was again somewhat lower than that previously reported for the trimethyl ester, *viz.* 154.5–155.5°. This melting point, however, was found to be easily depressed by slight impurities. A mixture of esters from both sources melted at 153–155°. The rotations of both samples were practically negligible.

The results of the analysis on the more recent trimethyl ester were as follows:

3.205 mg. substance: 2.095 mg. H_2O, 7.370 mg. CO_2.

$C_{25}H_{34}O_9$.	Calculated.	C 62.73, H 7.17
	Found.	" 62.71, " 7.30

In all other respects their properties were identical. A phenylhydrazone prepared from the new trimethyl ester melted at 197° as previously reported and showed no depression when mixed with the original phenylhydrazone.

4.761 mg. substance: 2.950 mg. H_2O, 11.425 mg. CO_2.
$C_{31}H_{40}O_8N_2$. Calculated. C 65.46, H 7.10
Found. " 65.39, " 6.93

Duodephanthondiacid—Here again the preparation of this acid from the above dimethyl ester was greatly improved by the substitution of piperidine for 0.1 N NaOH as previously described.[8] 2.5 gm. of crude undephanthontriacid dimethyl ester, as directly obtained, were dissolved in a mixture of 12.5 cc. of piperidine and 25 cc. of water. The solution was refluxed preferably in an atmosphere of nitrogen for 16 hours after which time the solution no longer gave a nitroprusside reaction. After concentration to dryness under diminished pressure the residue was redissolved in water. On acidification to Congo red the acid gradually crystallized. The amount obtained was 60 to 80 per cent of the weight of the dimethyl ester employed. After recrystallization from acetone it melted with effervescence at 264°. In all respects the substance proved to be identical with that previously described.

4.260 mg. substance: 2.835 mg. H_2O, 10.041 mg. CO_2.
$C_{21}H_{28}O_7$. Calculated. C 64.25, H 7.20
Found. " 64.28, " 7.45

For further characterization the dimethyl ester was prepared, as previously described, with diazomethane. The ester is characterized by its great crystallizing powers. It separated readily from methyl alcohol as sparingly soluble prisms which melted at 166°, and showed no depression when mixed with the ester originally described.

4.332 mg. substance: 2.988 mg. H_2O, 10.445 mg. CO_2.
$C_{23}H_{32}O_7$. Calculated. C 65.67, H 7.68
Found. " 65.76, " 7.72

The ester was also prepared by gently heating for 3 minutes at 70° a solution of the acid in 0.5 per cent dry methyl alcoholic hydrochloric acid. Longer heating resulted in a more deep-seated reaction which gave rise to obscure non-crystalline products.

Duodephanthondiacid Dimethyl Ester Ketazine—A solution of 0.1 gm. of duodephanthondiacid dimethyl ester in a few cc. of methyl alcohol was treated with an excess (over 3 mols) of a 3 per cent hydrazine solution which had been previously neutralized with acetic acid. On standing at ordinary temperature the mixture gradually deposited in excellent yield fine needles of the ketazine. After recrystallization from methyl alcohol it melted at 184°.

4.260 mg. substance: 2.935 mg. H_2O, 10.305 mg. CO_2.
6.182 " " : 0.194 cc. N (761 mm., 24°).

$C_{46}H_{64}O_{12}N_2$.	Calculated.	C 65.98, H 7.71, N 3.35
	Found.	" 65.98, " 7.70
	"	" 3.61

Reduction of Duodephanthondiacid—0.35 gm. of carefully recrystallized duodephanthondiacid in alcoholic solution was shaken with hydrogen and 0.1 gm. of Adams and Shriner's platinum oxide catalyst. After the initial reduction of the catalyst 1 mol of hydrogen was absorbed within 20 minutes, and then the reaction stopped. After concentration the reduction product readily crystallized. This substance proved to be the dilactone acid due to simultaneous lactonization. It formed prisms and narrow platelets from alcohol which melted at 253°.

4.380 mg. substance: 2.935 mg. H_2O, 10.735 mg. CO_2.

$C_{21}H_{28}O_6$.	Calculated.	C 66.99, H 7.50
	Found.	" 66.84, " 7.50

In another experiment the reaction was found to take a different course when a sample of duodephanthondiacid of different origin was employed. This material had been prepared by the previously described method in which undephanthontriacid dimethyl ester obtained by the permanganate oxidation of anhydroisostrophanthonic dimethyl ester was decomposed by refluxing with 0.1 N alkali. 0.7 gm. of this material in alcoholic solution was reduced with 50 mg. of platinum oxide catalyst and hydrogen. After concentration of the filtrate from the catalyst a crystalline residue was obtained which after recrystallization from a small volume of methyl alcohol yielded flat needles or narrow leaflets which melted at 221°. On recrystallization from methyl alcohol this

melting point was raised to 234°. Analysis and titration showed that the substance was the *hydroxy acid.*

4.642 mg. substance: 3.120 mg. H_2O, 10.895 mg. CO_2.

$C_{21}H_{30}O_7$. Calculated.	C 63.93,	H 7.67
Found.	" 64.01,	" 7.52

16.467 mg. of substance were dissolved in 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.795 cc. Calculated for 2 equivalents, 0.835 cc. The above solution was treated with 3 cc. of 0.1 N NaOH, refluxed for 4 hours, and then titrated back. Found, 0.460 cc. or a total of 1.255 cc. Calculated for 3 equivalents, 1.252 cc.

We have not investigated further the reason for the formation of the intermediate hydroxy acid in this case. Later attempts to duplicate the preparation of the hydroxy acid (or the hydroxy ester from the dimethyl ester as given below) always gave the dilactone. This fact was of much greater consequence in our problem.

Reduction of Duodephanthondiacid Dimethyl Ester—In repeated experiments with the dimethyl ester no duodephantholdiacid dimethyl ester could be obtained since lactonization with cleavage of methyl alcohol proceeded too quickly. The substance which resulted was the dilactone monomethyl ester.

0.27 gm. of the dimethyl ester was suspended in methyl alcohol and shaken with 0.1 gm. of platinum oxide and hydrogen. The sparingly soluble ester dissolved within about 20 minutes after the absorption of 1 mol of H_2. Concentration of the filtrate from the catalyst yielded the sparingly soluble monomethyl ester. Recrystallized from dilute acetone, it formed needles which melted at 240°. It is sparingly soluble in methyl and ethyl alcohols at ordinary temperature but is readily soluble in chloroform and acetone.

5.090 mg. substance: 3.585 mg. H_2O, 12.596 mg. CO_2.
5.300 " " : 3.260 " AgI.

$C_{22}H_{30}O_6$. Calculated.	C 67.65,	H 7.75,	OCH_3 7.95
Found.	" 67.48,	" 7.88	
"			" 8.12

15.819 mg. of substance were refluxed in 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 3 hours and then titrated back against phenol-

phthalein. Calculated for 3 equivalents, 1.216 cc. Found, 1.178 cc.

Unsaturated Lactone Anhydride, $C_{21}H_{24}O_5$, from Duodephanthondiacid—The attempt by means of methyl alcoholic hydrochloric acid to open the lactone group of duodephanthondiacid with simultaneous dehydration and esterification in order to form an unsaturated substituted succinic ester was unsuccessful, due to the non-crystalline character of the reaction products. Success was obtained by the use of acetic anhydride and acetyl chloride which, while giving an unsaturated substituted succinic anhydride, caused simultaneous lactonization of the carboxyl group on the carbonyl group.

2.7 gm. of duodephanthondiacid were heated in a sealed tube at 80° with a mixture of 30 cc. of acetic anhydride and 3 cc. of acetyl chloride. The sparingly soluble substance gradually dissolved when the mixture was shaken. After solution the heating was continued for 18 hours. On concentration under diminished pressure a crystalline residue remained. This was redissolved in chloroform and the solution was extracted with dilute sodium carbonate solution, then with water, and was finally dried. On careful addition of petrolic ether to the concentrated solution, platelets of the unsaturated lactone anhydride separated. The yield was 2.05 gm. When recrystallized from dilute acetone the substance forms long narrow plates which melted at 242° after preliminary sintering. It is readily soluble in chloroform and acetone and very sparingly soluble in methyl and ethyl alcohols, ether, and benzene. The substance does not give the Legal reaction with nitroprusside.

5.167 mg. substance: 3.250 mg. H_2O, 13.393 mg. CO_2.

$C_{21}H_{24}O_5$.	Calculated.	C 70.76, H 6.79
	Found.	" 70.68, " 7.03

The anhydride nature of the substance was shown by its behavior toward alkali. Although insoluble in dilute sodium carbonate solution it rapidly dissolved in dilute NaOH solution. On being boiled with alkali, 3 equivalents were consumed, 2 by the anhydride group and 1 by the lactone group.

15.102 mg. of substance were refluxed with 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 3 hours and then titrated back against

phenolphthalein. Calculated for 3 equivalents, 1.272 cc. Found, 1.258 cc.

Hydrogenation of the Unsaturated Lactone Anhydride, $C_{21}H_{24}O_5$, to the Saturated Desoxy Acid Anhydride, $C_{21}H_{30}O_5$—0.52 gm. of the unsaturated lactone anhydride was hydrogenated in acetic acid solution with 50 mg. of platinum oxide catalyst. A rapid absorption occurred which practically stopped after 20 minutes. After deduction for the reduction of the catalyst, the absorption amounted to 105 cc. (0°, 760 mm.). Calculated for 3 mols, 99 cc. On concentration to dryness under diminished pressure a glassy residue remained which crystallized rapidly when treated with dry ether. After standing, the crystals were collected with ether. On careful addition of petrolic ether to the solution in chloroform, the substance separated as needles which melted at 173° after slight preliminary softening. The yield of the crystalline acid was approximately one-third of the weight of the starting material. The major portion of reaction product remained in solution and on concentration remained as a resin which could not be made to crystallize. This material was unquestionably a mixture of low melting isomers which were simultaneously formed during the hydrogenation.

The acid character of the reaction product was readily seen by its ready solution in dilute carbonate in contradistinction to the unsaturated lactone anhydride. It is soluble in the usual organic solvents with the exception of petrolic ether. For analysis the substance was dried at 100° and 15 mm.

4.828 mg. substance: 3.620 mg. H_2O, 12.360 mg. CO_2.

$C_{21}H_{30}O_5$.	Calculated.	C 69.57, H 8.35
	Found.	" 69.82, " 8.39

Saturated Triacid, $C_{21}H_{32}O_6$—On warming the above anhydride acid with dilute alkali the anhydride group was readily saponified. On acidification an amorphous mass separated which gradually crystallized as delicate needles. The tribasic acid was recrystallized by the careful dilution of its solution in a small volume of acetone. It formed minute delicate needles which were difficult to filter. It did not exhibit a sharp melting point but slowly softened to a resin as the temperature rose above 120°.

For analysis the substance was dried at 100° and 15 mm.

5.197 mg. substance: 4.110 mg. H_2O 12.680 mg. CO_2.

$C_{21}H_{32}O_6$.	Calculated.	C 66.27, H 8.48
	Found.	" 66.55, " 8.85

Saturated Triacid Trimethyl Ester—The above triacid in ether solution was treated with diazomethane. On concentration a colorless oil remained which finally crystallized after long standing. Because of its solubility in most organic solvents petrolic ether was found most suitable for its collection and recrystallization. From its concentrated solution in this solvent it slowly separated as needles which melted at 62°.

4.920 mg. substance: 3.880 mg. H_2O, 12.300 mg. CO_2.
3.830 " " : 6.268 " AgI.

$C_{24}H_{38}O_6$.	Calculated.	C 68.20, H 9.07, OCH_3 22.01
	Found.	" 68.18, " 8.82, " 21.62

Unsaturated Lactone from Undephanthontriacid Dimethyl Ester—1.5 gm. of undephanthontriacid dimethyl ester were heated in a sealed tube with 30 cc. of acetic anhydride and 3 cc. of acetyl chloride at 80° for 15 hours. After concentration under diminished pressure a crystalline residue remained. This was dissolved in chloroform and the solution was extracted with dilute carbonate and then water. The unsaturated lactone was obtained on concentration of the chloroform solution. On recrystallization from acetone it formed needles which melted at 199°. Although it may be recrystallized from methyl alcohol this was found to be complicated by the ready cleavage of the unsaturated lactone group to form the neutral trimethyl ester of undephanthontriacid.

For analysis the substance was dried at 100° and 15 mm.

3.765 mg. substance: 2.355 mg. H_2O, 8.925 mg. CO_2.
3.903 " " : 4.100 " AgI.

$C_{24}H_{30}O_8$.	Calculated.	C 64.54, H 6.78, OCH_3 13.90
	Found.	" 64.65, " 7.00
	"	" 13.87

The unsaturated lactone gives a Legal reaction which is more pronounced than in the case of undephanthontriacid dimethyl ester.

Undeplogondiacid Monomethyl Ester—0.25 gm. of anhydroiso-

periplogonic methyl ester[7] dissolved in 25 cc. of acetic acid was oxidized by ozone under the conditions used with anhydroisostrophanthonic dimethyl ester. After removal of the solvent under diminished pressure, the resinous residue was treated with water and heated on the bath to decompose any ozonide. On cooling and standing, the oxidation product gradually crystallized. On careful dilution of the acetone solution it gradually formed a mass of delicate needles which melted at 182–184°. The substance was completely soluble in dilute ammonia. Contrary to the analogous strophanthidin derivative it gave no reaction with nitroprusside.

3.627 mg. substance: 2.457 mg. H_2O, 8.680 mg. CO_2.
3.755 " " : 2.137 " AgI.

$C_{23}H_{32}O_7$.	Calculated.	C 65.68, H 7.68,	OCH_3 7.38
	Found.	" 65.27, " 7.58	
	"		" 7.52

Unsaturated Lactone, $C_{23}H_{30}O_6$, from Undeplogondiacid Monomethyl Ester—The previous keto acid was heated at 80° for 16 hours in 10 parts of a mixture of acetic anhydride and acetyl chloride (10:1). After removal of the reagent under reduced pressure the residue was dissolved in chloroform. The solution was washed with dilute carbonate. The concentrated chloroform solution crystallized readily when treated with ether. On recrystallization from chloroform-ether it separated as broad prismatic needles which melted at 235–236°.

4.155 mg. substance: 2.750 mg. H_2O, 10.425 mg. CO_2.

$C_{23}H_{30}O_6$.	Calculated.	C 68.61, H 7.52
	Found.	" 68.43, " 7.41

This unsaturated lactone gives a faint, slowly developing, atypical Legal reaction which is not comparable with that usually given by $\Delta^{\beta,\gamma}$-lactones or by the analogous unsaturated lactone from undephanthontriacid dimethyl ester.

Part B

Saponification of the Saturated Lactone Dimethyl Ester, $C_{25}H_{36}O_7$, to the Saturated Triacid, $C_{23}H_{34}O_8$—The saturated lactone dimethyl ester, $C_{25}H_{36}O_7$, is a substance previously described.[5] Its origin

was as follows: β-Isostrophanthic lactone acid[9] yielded an unsaturated anhydroanhydride acetate, $C_{25}H_{30}O_7$, with acetic anhydride-acetyl chloride. This on saponification gave the dibasic anhydrolactone acid, $C_{23}H_{30}O_7$, which was converted into the so called β-dimethyl ester, $C_{25}H_{34}O_7$. This ester on hydrogenation gave the saturated lactone dimethyl ester which was used in the following experiments.

The complete saponification of the lactone ester to the triacid was complicated by the resistance to hydrolysis of the stable lactone group which is characteristic of substances of the β-isostrophanthic lactone acid series and the sparing solubility of the sodium salt of the dibasic acid first formed on saponification of the two methyl ester groups. Depending upon the conditions employed either result could be obtained.

0.1 gm. of the lactone dimethyl ester was refluxed in a mixture of 6 cc. of 10 per cent NaOH and 6 cc. of absolute alcohol. Solution was complete in 15 minutes. If the heating was continued longer under these conditions with the hope of saponifying the lactone group this was prevented by the gradual separation of the sparingly soluble sodium salt of the dibasic lactone acid. On dilution the salt redissolved and on subsequent acidification the free acid separated as platelets. From dilute acetone it formed diamond-shaped plates which frothed up at 258° after preliminary softening.

For analysis the substance was dried at 100° and 15 mm.

4.305 mg. substance: 0.322 mg. H_2O.

$C_{23}H_{32}O_7 \cdot 2H_2O$. Calculated. H_2O 7.89. Found. 7.48

3.983 mg. anhydrous substance: 2.770 mg. H_2O, 9.630 mg. CO_2.

$C_{23}H_{32}O_7$.	Calculated.	C 65.67,	H 7.68
	Found.	" 65.93,	" 7.78

For complete saponification to the tribasic acid the following procedure was used.

0.2 gm. of the lactone dimethyl ester was refluxed in a mixture of 5 cc. of pyridine, 15 cc. of 2 N NaOH, and 10 cc. of water for 4 hours. On acidification the acid separated first as a jelly which gradually crystallized. On recrystallization from dilute acetone it separated as compact masses which contained solvent and

effervesced at 225°. On drying at 100° and 15 mm. water was not completely removed.

4.704 mg. substance: 3.315 mg. H_2O, 10.700 mg. CO_2.

$C_{23}H_{34}O_8 \cdot \frac{1}{2}H_2O$.	Calculated.	C 61.70,	H 7.90
	Found.	" 62.04,	" 7.88

Trimethyl Ester, $C_{26}H_{40}O_8$—The ester, prepared with diazomethane, formed delicate needles from dilute methyl alcohol which melted at 199° and were easily soluble in the alcohols and acetone and appreciably in ether.

For analysis it was dried at 100° and 15 mm.

4.918 mg. substance: 3.710 mg. H_2O, 11.710 mg. CO_2.
4.360 " " : 6.250 " AgI.

$C_{26}H_{40}O_8$.	Calculated.	C 64.97,	H 8.40,	OCH_3 19.37
	Found.	" 64.95,	" 8.44,	" 18.92

Keto Ester, $C_{26}H_{38}O_8$—The above trimethyl ester was oxidized at ordinary temperature in acetic acid solution with Kiliani chromic acid solution. When recrystallized from methyl alcohol the ketone formed needles which melted at 220°.

4.470 mg. substance: 3.160 mg. H_2O, 10.740 mg. CO_2.

$C_{26}H_{38}O_8$.	Calculated.	C 65.24,	H 8.01
	Found.	" 65.52,	" 7.91

Repeated attempts to convert this substance into an anhydro compound resulted only in the formation of non-crystalline resins.

The oxime formed needles from methyl alcohol which melted at 210°.

4.390 mg. substance: 3.190 mg. H_2O, 10.208 mg. CO_2.

$C_{26}H_{39}O_8N$.	Calculated.	C 63.25,	H 7.97
	Found.	" 63.37,	" 8.13

β-Isostrophanthonic Dimethyl Ester—This was prepared from β-isostrophanthic dimethyl ester exactly as described in our earlier work with the α-ester.[10] The keto ester formed long narrow plates from methyl alcohol which melted at 248–250°. It is easily soluble

[10] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 819 (1927).

in chloroform and acetone, sparingly soluble in the alcohols, and insoluble in ether.

4.115 mg. substance: 2.730 mg. H_2O, 9.785 mg. CO_2.

$C_{25}H_{34}O_8$.	Calculated.	C 64.90, H 7.41
	Found.	" 64.85, " 7.42

The oxime crystallized from dilute methyl alcohol as needles which sintered above 128° and frothed up at 190°, then resolidified and remelted at 215–217°.

4.198 mg. substance: 2.890 mg. H_2O, 9.622 mg. CO_2.

$C_{25}H_{35}O_8N$.	Calculated.	C 62.86, H 7.39
	Found.	" 62.51, " 7.71

β-Isostrophanthonic ester was dehydrated as previously described in the case of the α-isomer,[10] with the exception that it is preferable to substitute methyl alcohol for ethyl alcohol to avoid "Umesterung." The anhydro compound formed rhombs from methyl alcohol which melted at 205–206° and were indistinguishable from the previously described anhydro-α-isostrophanthonic dimethyl ester. The melting point of the latter which was previously reported at 210° was found to vary. It was found after recrystallization to be 206°. No depression was obtained with the mixture.

The substance from the β-series showed a rotation

$[\alpha]_D^{20} = +72°$ (c = 0.555 in pyridine).

The rotation previously reported with the substance obtained from the α-series was:

$[\alpha]_D^{25} = +74°$ (c = 1.023 in pyridine).

4.205 mg. substance: 2.720 mg. H_2O, 10.403 mg. CO_2.

$C_{25}H_{32}O_7$.	Calculated.	C 67.53, H 7.26
	Found.	" 67.47, " 7.24

Part C

The Oxidative Cleavage of Desoxy-α-Isostrophanthonic Dimethyl Ester to the Dibasic Acid, $C_{25}H_{34}O_{10}$—In more recent experiments on the preparation of desoxyisostrophanthonic dimethyl ester[2] by hydrogenation of anhydro-α-isostrophanthonic dimethyl ester, the more sparingly soluble isomer which separated as stout prisms or rhombs was always found to be the preponderating reaction

product. A number of modifications of the method of hydrogenation did not alter the result. This isomer, for which a rotation was found of $[\alpha]_D = -4°$ (c = 0.7 in pyridine), was found on rechecking to give $[\alpha]_D = +8°$ (c = 0.5 in pyridine). On oxidation of this ketonic ester with CrO_3 no crystalline reaction product could be obtained. The other more soluble isomer which was always formed in much smaller amount gave a crystalline dibasic acid as follows:

0.5 gm. of the desoxy keto ester of $[\alpha]_D = +57°$ (c = 0.7 in pyridine) was dissolved in a mixture of 10 cc. of acetic acid and 1 cc. of H_2O. 2 cc. of Kiliani CrO_3 solution were added and the mixture was heated at 70° for 1 hour. The diluted mixture was extracted with chloroform. The extract after repeated washings with water to remove excess acetic acid was extracted with dilute ammonia. The concentrated aqueous solution on acidification gave a copious separation of the acid which gradually crystallized. This dibasic acid formed needles from dilute acetone which melted at 191–193°.

4.692 mg. substance: 2.985 mg. H_2O, 10.512 mg. CO_2.
4.235 " " : 4.000 " AgI.
$C_{25}H_{34}O_{10}$. Calculated. C 60.70, H 6.93, OCH_3 12.55
Found. " 61.10, " 7.12
" " 12.48

When an attempt to distil this acid under very low pressure was made in order to obtain a possible pyroketone, decomposition occurred and no crystalline reaction product could be obtained.

A small amount of unchanged keto ester was recovered from the neutral fraction of the above oxidation mixture.

Anhydride, $C_{25}H_{32}O_9$—The above dibasic acid was heated at 80° for 16 hours in a sealed tube with 10 parts of an acetic anhydride-acetyl chloride mixture (10:1). On evaporation under diminished pressure a residue remained which readily crystallized under ether. The anhydride separated on addition of ether to its chloroform solution as micro leaflets which melted at 256° with decomposition.

3.488 mg. substance: 2.192 mg. H_2O, 8.037 mg. CO_2.
3.773 " " : 3.800 " AgI.
$C_{25}H_{32}O_9$. Calculated. C 62.99, H 6.77, OCH_3 13.03
Found. " 62.84, " 7.03
" " 13.30

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APPARATUS TO CIRCULATE LIQUID UNDER CONSTANT PRESSURE IN A CLOSED SYSTEM

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This apparatus is designed to circulate a liquid and to maintain a constant pressure in a sterile system, without the use of joints or moving parts in contact with the circulating liquid.

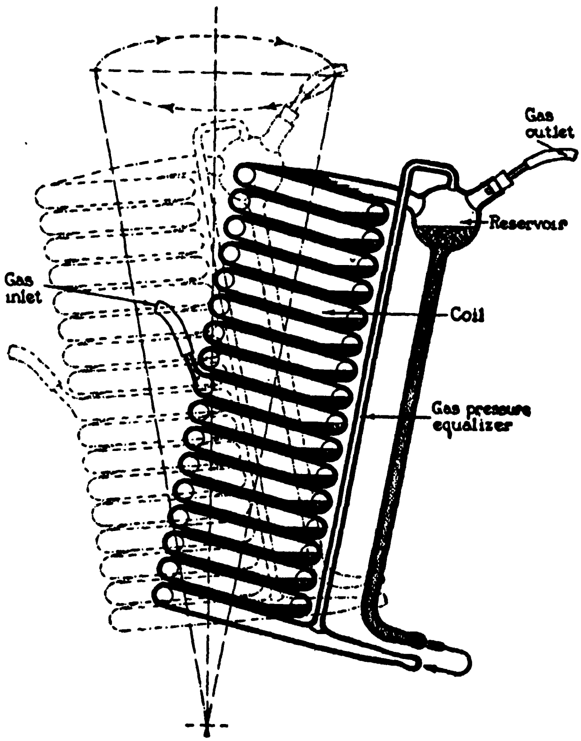

Diagram to show basic principles of apparatus

The apparatus is a single piece of glass. Pressure is maintained by the head of liquid and the liquid is raised and kept in circulation by placing the apparatus on a tilted base which is given a circular motion

without being permitted to rotate. This motion carries the liquid up the coil and into the top reservoir. Gases can be introduced through the tube half way up the coil, and an internal pressure can be maintained, if desired, by the displacement of water or other fluid by the exhaust gases.

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PROTEIN COAGULATION AND ITS REVERSAL

THE IDENTITY OF NORMAL HEMOGLOBIN WITH THE HEMOGLOBIN PREPARED BY THE REVERSAL OF COAGULATION, AS DETERMINED BY SOLUBILITY TESTS

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INTRODUCTION

Experiments which lead to the conclusion that the coagulation of proteins is reversible have been presented in previous papers (review and literature in Anson and Mirsky, 1931). From coagulated hemoglobin, globin, and serum albumin one can again prepare soluble, crystalline protein. In order to prove that coagulation is reversible it is necessary to show that the soluble protein finally obtained from completely coagulated protein is identical with the original native protein. Actually, within the limits of error, "reversed" hemoglobin has the same temperature of coagulation, the same spectrum, and the same gas affinities as normal hemoglobin and, although no exact measurements have been made, it appears to have the same crystalline form (Mirsky and Anson, 1929). The structural changes which take place during denaturation and its reversal are not completely known. It is accordingly desirable to have some means of detecting any possible difference of unknown nature between normal and "reversed" hemoglobin. Since the solubility of a protein depends on all of its groups, if "reversed" and native hemoglobin were different in any way, then theoretically their solubilities ought to be different. The experiments to be described show that within less than two per cent their solubilities are the same. Furthermore, native globin has been prepared from coagulated globin and the hemogobin synthesized from this "reversed" globin and heme has the same solubility as normal hemoglobin.

There is no normal globin with which "reversed" globin could be directly compared because globin is denatured in the process of its preparation from hemoglobin. Finally, the solubility of a mixture of solid normal, "reversed" and synthetic hemoglobins is the same as the solubility of any one of these proteins alone.

Method

In general, in making a solubility measurement, it is necessary first to prepare a suspension of the solid in a solvent of definite composition and, secondly, to saturate the solvent with the solid. With an unstable protein such as methemoglobin,[1] unless the procedures are carried out quickly and gently the protein changes in solubility during the experiment itself. To avoid such changes as much as possible the present measurements have been made with amorphous methemoglobin which is formed and dissolved much more readily than crystalline methemoglobin. The use of the amorphous form of methemoglobin, however, introduces a new technical difficulty. It is a familiar fact, although we have not found any quantitative study of it, that any solid is more soluble when amorphous than when crystalline. In the case of methemoglobin the difference is extreme. A saturated solution of amorphous methemoglobin is some fifty times supersaturated in respect to crystalline methemoglobin.[2] With time, therefore, methemoglobin crystallizes out of a saturated solution of the amorphous protein. The measurement of the solubility of amorphous methemoglobin must be finished before this crystallization has taken place to any significant extent.

[1] Methemoglobin has been used rather than the more stable carbon monoxide hemoglobin because it is methemoglobin which is obtained on the reversal of coagulation. The preparation of pure carbon monoxide hemoglobin from methemoglobin presents greater experimental difficulties than does the instability of methemoglobin.

[2] Methemoglobin has about the same solubility as oxyhemoglobin. Oxyhemoglobin has been supposed to be less soluble only because the solubility of the crystalline form of oxyhemoglobin has been compared with that of the amorphous form of methemoglobin. Under suitable conditions methemoglobin can be crystallized with ease in any amount from dilute or concentrated, isoelectric or not isoelectric solutions.

Preparation of Suspension.—The preparation of the suspension of amorphous methemoglobin in a solvent of definite composition is a simple matter. First the hemoglobin is brought to a definite hydrogen ion concentration by the addition of a phosphate buffer. Conditions are made as favorable as possible to secure this result. The protein is first freed of salts by dialysis. The buffer used has almost the same hydrogen ion concentration as the dialyzed protein, which fortunately is also the hydrogen ion concentration at which the buffer has its maximum buffering efficiency. And the concentration of the buffer is relatively high while that of the protein is relatively low. After the protein has been brought to a definite hydrogen ion concentration, it is salted out by the addition of concentrated ammonium sulfate. If the solutions are first cooled, it is possible to mix them completely before any precipitation whatsoever takes place. When the temperature is raised to 25°C. almost all the protein precipitates in the form of very fine particles which do not settle. The solvent held by the precipitate is the same as that in the bulk of the solution. This condition, in most solubility experiments, is achieved only by long and repeated washings; a procedure unsuitable for unstable proteins and out of the question if the crystallization of methemoglobin is to be avoided.

Equilibration.—The suspension which has just been described is formed by the precipitation of hemoglobin from a supersaturated solution. In the presence of a large excess of so fine a precipitate the solution cannot remain supersaturated. After only a few minutes gentle shaking the solution is in equilibrium with the solid. To make sure that equilibrium has been reached, the equilibrium is in every case also approached from below by shaking the suspension with an equal volume of solvent. The hemoglobin content of the filtrate is the same whether the equilibrium is approached from above or below.

Avoidance of Crystallization.—Finally, we shall state the precautions taken to avoid crystallization of methemoglobin from the saturated solution of amorphous methemoglobin and the evidence that in fact such crystallization does not take place. The salt concentration of the solvent is so chosen that the solubility of the protein is only 0.4 mg. per ml. From so dilute a solution crystallization is slow. In time, nevertheless, crystallization does take place from even a dilute solution. The filtration, therefore, must not take too long. This means that

the suspension of precipitate must not be too concentrated, for the more concentrated the suspension, the slower the filtration. With the less than 1 per cent suspension used filtration is sufficiently fast. It is possible to have a dilute suspension and still have the large excess of solid needed for quick equilibration because the solubility is made extremely low.

That no significant amount of crystallization takes place under the conditions chosen to make crystallization relatively slow and filtration relatively fast is shown by the facts that successive portions of filtrate have the same methemoglobin concentration and that the same solubility value is obtained whether the equilibrium is approached from above or from below. When visible crystallization does begin, successive portions of filtrate contain less and less protein, and less and less protein dissolves when the suspension is shaken with solvent. It may be that small, non-detectable amounts of hemoglobin crystals are formed even during the experiment but that amorphous hemoglobin dissolves to maintain the equilibrium just as fast as the solution loses hemoglobin by crystallization.

RESULTS

As may be seen from the table, the solubilities of normal, "reversed," and synthetic hemoglobins and of the mixture of all three solids are the same within 2 per cent. In general, even small differences between two proteins are reflected in differences in solubility much greater than 2 per cent. The solubilities of horse and ox hemoglobins are so different they cannot be measured under the same conditions. It is not excluded, nevertheless, that normal and "reversed" hemoglobins are slightly different and have slight differences in solubility which fall within the experimental error. The problem of the solubilities of slightly different proteins, such as horse and mule hemoglobins, or the different derivatives of horse hemoglobin, will be discussed experimentally in a later paper. What one can conclude from the present result is that if normal and "reversed" hemoglobins are not completely identical, they are, at least, nearly the same. With the methods now available it is not possible to prove beyond doubt that any two proteins are completely identical. All one can show is that they cannot be distinguished by certain specific tests.

It remains to discuss the significance or rather the lack of significance of the measurement of the mixed solubility of normal, "reversed," and synthetic hemoglobins. According to the phase rule the solubilities of different solids suspended together in the same solvent are independent; the total amount of substance dissolved is equal to the sum of the individual solubilities of the solids. This is true regardless of how small the differences are between the solids provided only that the substances are present as individual solid phases. The fact, then, that the solubilities of normal, "reversed," and synthetic hemoglobins are not additive might be considered as proof that these three hemoglobins do not differ even slightly. Actually, however, as will be seen later, slightly different amorphous hemoglobins do act on one another to form solid solutions.[3] In a suspension of three slightly different hemoglobins there is only one solid phase, the solid solution of the three hemoglobins. The solubility observed is that of this one phase,

TABLE

	Normal	Reversed	Synthetic	Mixed
Solubility, mg. Hb per ml. filtrate..........	4.09	4.08	4.05	4.00

not the sum of the individual solubilities of the three hemoglobins. The experiment on the solubility of the mixture of normal, "reversed," and synthetic hemoglobins does *not* provide, therefore, any additional evidence for their identity.

EXPERIMENTAL

Preparation of Hemoglobin.—Distilled water is added to washed horse corpuscles to make the hemoglobin concentration about 12 per cent. The solution is shaken vigorously with a fifth its volume of toluol and left overnight in the cold. The upper layer of toluol and stromata is then removed, and the rest shaken with a seventh of its volume of centrifuged alumina cream (Tracy and Welker, 1915), allowed to stand an hour or more and filtered. Finally to avoid crystallization the hemoglobin is diluted to 10 per cent and stored in the cold under toluol.

If carbon monoxide hemoglobin is desired the blood is immediately saturated

[3] Evidence suggesting that the crystalline forms of slightly different hemoglobins form solid solutions has already been given by Landsteiner and Heidelberger, (1923).

with carbon monoxide and the solution is always kept under carbon monoxide. There are slight differences in the preparation of horse and ox hemoglobins. Horse corpuscles settle from serum of their own accord in a few hours while ox corpuscles have to be centrifuged. Horse corpuscles can be separated from sodium chloride solution with a Sharples centrifuge without any large proportion being broken, while ox corpuscles are broken by this procedure. A twentieth of the total volume of alumina cream suffices to secure good filtration of ox hemoglobin while much more is needed to secure complete and immediate separation of the toluol globules from horse hemoglobin solutions.

Nitrogen Estimation.—The Kjeldahl determinations are done with mercuric oxide as catalyst. As Dr. Northrop has observed, complete digestion of hemoglobin is not obtained with copper. Hemoglobin foams much more than do other common proteins. With small Kjeldahl flasks it is impossible to obtain accurate results unless this foaming is avoided. The Kjeldahl flask containing the solution to be estimated plus a drop of sulfuric acid may be left in an oven until practically all the water is evaporated;[4] or all the sulfuric acid may be added to the hemoglobin and the acid solution left in the oven until the hemoglobin has been hydrolyzed past the foaming stage. It is better to use the smaller amount of acid if the flask is to be left in the oven a short time, and the larger amount of acid if the flask is to be left in the oven a long time.

Preparation of Normal Methemoglobin.—To each 100 ml. of 10 per cent carbon monoxide hemoglobin is added at room temperature 0.7 gm. powdered potassium ferricyanide. The resulting methemoglobin is dialyzed overnight in the cold in a shaking dialyzer (Kunitz and Simms, 1928) against running distilled water. If a 15 per cent solution of methemoglobin is dialyzed a good deal of the protein crystallizes out.

Preparation of "Reversed" Methemoglobin.—The procedure is practically the same as that already described (Mirsky and Anson, 1930). To a 10 per cent solution of carbon monoxide hemoglobin at 5°C. is added three times its volume of 1/15 N HCl.[5] 3 minutes later 15 ml. of 0.1 N NaOH is added slowly with stirring to each 40 ml. of acid hemoglobin solution. After the partially neutralized solution has stood half an hour or more at room temperature the neutralization is completed by the addition of 5 ml. of 0.1 N NaOH for every 15 ml. already added.

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[4] In the test tube digestion of proteins with a mixture of sulfuric and phosphoric acids preliminary to direct Nesslerization, serious foaming occurs with all the proteins we have tried. Evaporation before digestion simplifies the procedure greatly especially if a large number of determinations are being made. If time is not important slow, partial digestion at 100°C. is also desirable before the final completion of digestion at boiling temperature. At 100°C. the phosphoric acid does not attack Pyrex glass.

[5] Through error, it was stated on page 478, line 10 of a previous paper (Mirsky and Anson, 1930) that an equal volume of acid was added. In other parts of that paper the solution was described correctly.

The solution is half saturated with ammonium sulfate and the precipitated denatured protein filtered off. To each 100 ml. of filtrate is added 16 gm. of solid ammonium sulfate. The resulting amorphous precipitate is filtered off and dialyzed against distilled water. The "reversed" methemoglobin may be crystallized either by adding 7.5 gm. instead of 16 gm. of ammonium sulfate to each 100 ml. of the solution half saturated with ammonium sulfate or by making a concentrated saturated solution of the amorphous hemoglobin.[6]

Preparation of Synthetic Methemoglobin.—To each 100 ml. of a 5 per cent solution of the acid acetone powder of denatured globin (Anson and Mirsky, 1930) is added first 48 ml. of 0.1 N NaOH and after 20 minutes 16.5 ml. more. The precipitate of denatured globin is filtered off. To each 100 ml. of the filtrate, which contains the native globin prepared by the reversal of coagulation and the denatured globin not precipitated by neutralization, is added 0.106 gm. of recrystallized hemin (Eastman) dissolved in three equivalents of NaOH. This amount of hemin could convert into hemoglobin all the globin contained in the original preparation. Half saturation of the solution with ammonium sulfate now precipitates any remaining denatured globin and any free heme not combined with globin. The rest of the procedure is the same as with "reversed" methemoglobin. Synthetic like "reversed" methemoglobin may be completely crystallized.

The Phosphate and Ammonium Sulfate Solutions.—The stock 3.6 M phosphate solution contains per liter 313.7 gm. K_2HPO_4 and 245.1 gm. KH_2PO_4. To get a 1.25 M PO_4 solution, 132.4 gm. of water are added to 100.0 gm. of 3.6 M PO_4. The 2 per cent methemoglobin solution contains 2 gm. (1.5 ml.) of hemoglobin to 113.5 gm. (100.0 ml.) of 1.25 M PO_4. The ammonium sulfate solution contains 533 gm. per liter. This about corresponds to a saturated solution at 15°C., a temperature which the solution may reach during the night. Since the same salt solutions were used in any given group of solubility measurements and only comparative values were desired, no check was made on the purity or dryness of the salts.

To precipitate the methemoglobin 10 gm. ammonium sulfate solution are added to each 6 gm. of 2 per cent hemoglobin in 1.25 M PO_4. The solvent then contains 10 gm. ammonium sulfate solution to each 5.90 gm. of 1.25 M PO_4.

All weighings are made easily and rapidly on a Curie balance to one part in five hundred. There is no significant loss or gain of water during the weighings. The solutions for the solubility experiment itself are weighed out in 50 ml. Pyrex Erlenmeyer flasks.

Mixing, Shaking, and Filtering.—The solutions of 2 per cent hemoglobin and ammonium sulfate are cooled in ice water. If the ammonium sulfate solution is left cold too long the salt will of course crystallize out. The flasks are removed from the ice water, dried, and their edges covered with thin films of vaseline.

[6] This is a sure and general way of crystallizing any protein whose amorphous and crystalline forms have very different solubilities. It works very well with serum albumin.

The solutions are mixed by being poured from one flask to the other and the mixed solution is brought to 25°C. and rocked gently for 8 minutes. Whether shorter times would suffice to secure equilibrium was not tried. Finally the solution is filtered through a folded No. 42 Whatman paper 7 cm. in diameter, the funnel being covered with a small Petri dish. The first 3 ml. portion is rejected because of adsorption of the hemoglobin by the filter paper. The next two portions are collected for the estimation of hemoglobin.

Estimation.—The hemoglobin is estimated colorimetrically as cyan methemoglobin. A blue glass is placed in the eyepiece of the colorimeter to make the estimation of the red pigment easier and more sensitive. The methemoglobin is converted into cyan methemoglobin by the addition of a trace of solid KCN.

CONCLUSIONS

1. Methemoglobin prepared from coagulated hemoglobin by the reversal of coagulation has the same solubility within 2 per cent as normal methemoglobin.

2. Methemoglobin synthesized from hemin and the native globin prepared by the reversal of coagulation of globin likewise has the same solubility as normal methemoglobin.

We are indebted to Dr. John H. Northrop for his helpful suggestions.

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PROTEIN COAGULATION AND ITS REVERSAL

GLOBIN

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It has already been shown that the coagulation of hemoglobin is reversible (review and literature in Anson and Mirsky, 1931). Hemoglobin, however, is a conjugated protein consisting of the simple colorless protein, globin, combined with the red iron porphyrin complex, heme. It might be supposed that the result obtained from the study of the reversibility of the coagulation of hemoglobin reflects not a property of proteins in general but a peculiarity which hemoglobin displays because it contains heme. We have accordingly studied the simple protein, globin, itself. If acid acetone under certain conditions is added to hemoglobin, the heme and the globin are separated, the heme remaining in solution and the globin being precipitated. By a suitable neutralization procedure it is possible to obtain from this acid acetone globin, with a yield of about 65 per cent, soluble, heat coagulable globin which can combine with heme to form crystallizable hemoglobin (Anson and Mirsky, 1930). The facts that acid acetone is in general a denaturing agent and that the denaturation of hemogobin can be reversed by exactly the same kind of neutralization procedure used to prepare soluble globin (Mirsky and Anson, 1930) suggest that the preparation of soluble globin from acid acetone globin consists essentially in the reversal of denaturation. In confirmation of this view, the present experiments show that acid acetone globin is denatured globin and that soluble globin can be prepared from globin which has been precipitated with trichloracetic acid or heated. The opinion of Hill and Holden (1927) that the preparation of soluble globin depends solely on the avoidance of denaturation is shown not to be justified by their experiments.

Evidence That Acid Acetone Globin is Denatured Globin.—Native proteins in general are soluble at their isoelectric points. Denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali. The solubility of acid acetone globin is that of a denatured protein. As prepared from hemoglobin acid acetone globin is a compound of globin with hydrochloric acid which dissolves in water to give a strongly acid solution. It is completely precipitated by rapid, complete neutralization of the acid with a buffer salt and almost completely precipitated by rapid neutralization with NaOH. For instance, if an equal volume of 1 M K_2HPO_4 is added to a 5 per cent solution of acid acetone globin, the filtrate gives only a slight haze with trichloracetic acid. It is *a priori* possible that denatured proteins when precipitated carry down any native, soluble protein present and that therefore complete precipitation at the isoelectric point is not proof of complete insolubility and denaturation. Experimentally, however, we have not been able to obtain evidence that any significant amount of carrying down takes place under the conditions of our experiments. When denatured hemoglobin is precipitated in the presence of native hemoglobin, the concentration of native hemoglobin in solution is not changed (Anson and Mirsky, 1929). Similarly, if one adds ammonium sulfate at 0°C. to a mixture of heat-denatured globin (heated in a solution acid enough to prevent precipitation) and unheated globin in acid, all the heated globin is precipitated and all the unheated globin remains in solution. Northrop (1930) has done essentially the same experiment and obtained the same result with known mixtures of native and denatured pepsin globulin, and he has shown further that when a given fraction of pepsin is denatured by alkali the fraction denatured as estimated by loss of enzyme activity without any precipitation whatsoever, is the same as the fraction denatured as estimated by the amount of protein nitrogen remaining in solution after the denatured pepsin has been precipitated at the isoelectric point. These experiments do not mean that coagulated protein cannot adsorb native protein at all. Such adsorption does take place. But it is significant in amount only when the concentration of coagulated protein is very great in comparison with that of the native protein.

In general when proteins are denatured there becomes free a number of SH and S-S groups which is equivalent to the total number of cysteine

and cystine groups in the protein.* The acid acetone globin has the number of free SH and S-S groups characteristic of denatured proteins (unpublished experiments). Meldrum and Dixon (1930) state that denatured globin has no sulfhydryl groups. A simple qualitative test with nitroprusside shows that such groups are in fact present.

Finally, if the native globin obtained from acid acetone globin were simply native globin which had escaped denaturation, then the further treatment of the globin with trichloracetic acid or heat, both well known denaturing agents, ought to result in further denaturation and hence in a much lower yield of soluble globin. In fact, as will now be seen, trichloracetic acid and heat have little effect on the yield.

Reversal Experiments with Globin Precipitated by Trichloracetic Acid.—If the acid acetone globin is rapidly completely neutralized, as has just been seen, it is almost completely precipitated. If it is first only partially neutralized, a little less than enough alkali to cause incipient precipitation being added, then about two-thirds of the globin is found to be soluble native globin when after a time the neutralization is completed (Anson and Mirsky, 1930). If enough alkali is first added to precipitate and just redissolve the globin, then about one-third of the globin is found to be soluble in 0.4 saturated ammonium sulfate. Reversal on the alkaline side of the isoelectric point with globin gives a lower yield than reversal on the acid side. Since trichloracetic acid denatures proteins in acid solution, in the presence of trichloracetic acid only the alkaline reversal procedure is possible. When this is tried with a trichloracetic precipitate the same 33 per cent yield is obtained as from the globin to which no trichloracetic acid has been added. The quantities used are as follows. To 15 ml. of a 3.3 per cent of acid acetone horse globin is added 25 ml. of 2.2 per cent trichloracetic acid. The centrifuged precipitate is suspended in 10 ml. water and dissolved with 13 ml. of 0.1 N NaOH. After a few minutes 0.1 N HCl is added to give a heavy precipitate and the solution is 0.4 saturated with ammonium sulfate. The yield is estimated by the procedure already described (Anson and Mirsky, 1930).

Experiments exactly the same as the one just described can be done with hemoglobin (Mirsky and Anson, 1930) and serum albumin (unpublished experiments).

* See Mirsky, A. E., and Anson, M. L., 1930, *Proc. Soc. Exp. Biol. and Med.*, **28**, 170.

Reversal Experiments with Heated Globin.—A 5 per cent acid solution of the acid acetone ox globin is heated in boiling water for 3 minutes. By the neutralization procedure a 60 per cent yield of soluble globin is obtained. Again the same experiment can be done with hemoglobin and serum albumin.

Soluble globin can likewise be obtained from acid acetone globin prepared from hemoglobin heat coagulated in neutral solution.

Hill and Holden's Theory.—After it was shown (Anson and Mirsky, 1925) that globin prepared by the classical method of Schulz (1898) is denatured globin and that hemoglobin is a compound of native globin and heme while the hemochromogen prepared from hemoglobin is a compound of denatured globin and heme, Hill and Holden (1927) devised a method for preparing native globin. They assumed to begin with that coagulation cannot be reversed and that therefore the mere fact that they obtained native globin was in itself *a priori* proof that their native globin had never been denatured. They did not carry out any experimental tests to see whether the globin in fact had escaped denaturation. Their procedure involved extreme precautions to avoid denaturation, such as the careful maintenance of low temperature during the separation of globin from hemoglobin, the use of an atmosphere free from organic materials, etc. These precautions supposedly were the essential elements of the procedure. Actually these precautions are entirely unnecessary. We have found that by dialyzing globin against cold distilled water one can get the same 60 per cent yield of soluble protein whether one carries out Hill and Holden's procedure for separating heme and globin in the cold or at room temperature. The 60 per cent yield obtained at room temperature is higher than the one Hill and Holden reported for their preparation in the cold. And the yield they reported was higher than the one they obtained since in estimating how much denatured protein remained they did not precipitate the denatured protein completely. The opinion that the denaturation which admittedly takes place in acid at room temperature prevents the obtaining of soluble globin on neutralization is therefore not correct. The differences in yields in the different preparations are due to different conditions of neutralization, not to the greater or less avoidance of denaturation. Since there is no evidence in Hill and Holden's experiments that they avoided dena-

turation and since the present experiments show that one can obtain soluble globin from globin which has been denatured, it is not decided by Hill and Holden's experiments whether or not they were correct in their theory that native globin can be separated directly from hemoglobin. An observation of Holden and Freeman (1928) suggests, however, that Hill and Holden did denature their globin. If Hill and Holden's acid solution of globin is 1/100 saturated with ammonium sulfate then no soluble globin is obtained on neutralization. To explain this fact, Holden and Freeman had to assume that, contrary to experience, this small amount of salt actually brings about a rapid denaturation of the protein. A simpler explanation and one in accord with experience, is that the salt precipitates the already denatured protein, and thereby prevents reversal of denaturization.

CONCLUSIONS

1. The globin prepared from hemoglobin by the acid acetone method is denatured globin.
2. The denaturation and coagulation of globin by acid acetone are reversible.
3. Soluble globin can be obtained from the acid acetone globin even if the globin is first precipitated by trichloracetic acid or heated to 100°C.
4. Hill and Holden's theory that they separated native globin from hemoglobin without any intermediate denaturation is not proven by their experiments.

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PROTEIN COAGULATION AND ITS REVERSAL

SERUM ALBUMIN

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The reversal of the denaturation and coagulation of hemoglobin and globin has been described in previous papers (*cf.* review and literature in: Anson and Mirsky, 1931). This paper describes the preparation of crystalline, soluble, native protein from coagulated serum albumin and discusses the significance of the fact that this preparation offers so little difficulty.

Relation of the Ease of the Reversal of Denaturation to the Solubility of the Denatured Protein

All denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali. There are, however, considerable differences in the solubilities of different denatured proteins just as there are in the solubilities of different native proteins. Denatured hemoglobin is much less soluble than denatured serum albumin. In the absence of salts it is precipitated over a much wider range of hydrogen ion concentration around the isoelectric point than is denatured serum albumin and it is precipitated from acid solution by a concentration of salt which does not precipitate denatured serum albumin. Denatured egg albumin is even less soluble than denatured hemoglobin. Not only is it insoluble over a wide range of hydrogen ion concentration, but even when the denatured egg albumin appears to be dissolved by small amounts of acid or alkali there is aggregation or invisible precipitation of the protein (unpublished viscosity experiments).

Corresponding to these differences in solubility are differences in the ease of the reversal of denaturation. The denaturation of the relatively soluble serum albumin can be reversed with great ease; the denaturation of the less soluble hemoglobin can be reversed only by taking special precautions; while so far it has not been possible to reverse the denaturation of the very insoluble egg albumin at all.[1] Some 75 per cent of denatured serum albumin is converted into soluble native serum albumin on neutralization of an acid solution of the denatured protein. It makes no difference in the yield whether the protein is rapidly brought to the isoelectric point in one step, or first allowed to stand for half an hour in a solution just acid or alkaline enough to prevent precipitation. The same high yield is also obtained if the acid solution is one-tenth saturated with ammonium sulfate. In contrast, if an acid solution of denatured horse hemoglobin is rapidly brought to the isoelectric point, practically all the protein is precipitated. Reversal of denaturation is obtained, however, if the protein is first allowed to stand in a solution just acid or alkaline enough to prevent denaturation. Even with this procedure, no reversal is obtained and all the protein is precipitated, if the acid solution is one-tenth saturated with ammonium sulfate (Anson and Mirsky, 1925, 1929, 1930; Mirsky and Anson, 1929, 1930*a*). Finally, in the case of the very insoluble egg albumin, there is no reversal of denaturation or neutralization no matter how the neutralization is carried out.

Experimentally, then, there are two different results of neutralization of an acid solution of denatured protein: precipitation or aggregation of the insoluble denatured protein, and conversion of the insoluble protein into soluble native protein by the reversal of denaturation. In the three cases studied the more soluble the denatured protein is under the neutralization conditions, the easier and more complete is the reversal of denaturation.

The fact that in the cases of serum albumin, hemoglobin, and egg albumin, reversal of denaturation is more difficult the more insoluble the denatured protein does not mean that solubility is the only factor which determines to what extent reversal takes place or whether it can

[1] Recent experiments, which remain to be confirmed, indicate that the denaturation of egg albumin can under certain conditions be reversed.

take place at all. Unfortunately the theory of the mechanism of the reversal of denaturation (like the theory of the mechanism of denaturation itself) is as yet in an unsatisfactory state. It is not clear why reversal should result at all on neutralization, since native proteins are unstable at their isoelectric points.[2] For instance, isoelectric methemoglobin or egg albumin coagulates slowly on standing, and rapidly on being shaken with air or toluol globules. Nor is it clear why it should not be possible to reverse denaturation completely, at least on repetition of the reversal procedure. If the reversal procedure is carried out with denatured hemoglobin, 65 per cent of the protein is converted into a soluble form while 35 per cent remains insoluble. If the reversal procedure is repeated with the part that remained insoluble, this time much less than 65 per cent of the protein is made soluble. The solution of the non-reversed fraction does not behave the same as the solution of the original denatured hemoglobin.

A possible factor in causing this incompleteness of reversal is secondary irreversible change in the protein caused by the denaturation procedure but distinct from denaturation. In the case of hemoglobin there is not as yet any experimental evidence of such secondary change. It is known, however, that when egg albumin is heated there is, entirely apart from the formation of insoluble protein, a splitting off of ammonia (Sørensen and Sørensen, 1925). Particularly interesting experiments have been done with pepsin. Kühne (1877) discovered that pepsin is inactivated by alkali. Goulding, Borsook, and Wasteneys (1926) showed that there are two different kinds of inactivation by alkali, a rapid kind which does not go to completion except in strong alkali, but the *extent* of which depends on the pH; and a slow kind which always goes to completion, but the *rate* of which is proportional to the hydroxyl ion concentration. Northrop (1930; 1931), working for the first time with the pure pepsin protein, showed that both kinds of inactivation are accompanied by a formation of protein insoluble at the isoelectric point. The rapid inactivation and formation of insoluble protein, however, can be partially reversed by a procedure identical with that used for the reversal of denaturation, while by the

[2] It must be remembered in this connection that surface coagulation is most rapid at the isoelectric point. Under the conditions for reversal, it is much slower, indeed may not take place at all.

same procedure the slow kind of inactivation cannot be reversed at all. The simplest explanation of these results is that the rapid inactivation is simply denaturation of the protein, while the slow inactivation is a secondary irreversible change, perhaps a hydrolysis.

Evidence That the Protein Has Been Denatured

Insolubility.—To prove that denaturation has been reversed it must be proved that the protein has been denatured in the first place. The serum albumin used in the neutralization experiments is prepared by the addition to native serum albumin of acid acetone which is, in general, an effective denaturing agent. Usually one can demonstrate that protein treated with acid acetone is denatured by showing that the protein is completely precipitated when brought to the isoelectric point. The relatively soluble denatured serum albumin, however, seems to be converted into native protein by just this procedure. This difficulty may be avoided by neutralizing at a temperature at which reversal does not take place. If native serum albumin is heated in acid solution, the protein is denatured but is kept in solution by the acid. If the acid is neutralized while the solution is still hot, the protein is completely precipitated. This precipitate of insoluble and hence denatured serum albumin may be separated, dissolved, and converted, with a yield of 65 per cent, into protein soluble in half saturated ammonium sulfate.

Effect of Heating and Trichloracetic Acid on Yield.—Were 75 per cent of the acid acetone serum albumin undenatured and were there no reversal on neutralization, then heating the acid acetone protein or precipitating it with trichloracetic acid ought to result in further denaturation and hence in a lowering of the yield of soluble protein on neutralization. Actually heating and trichloracetic acid have no effect on the yield, indicating that denaturation by acid acetone has been complete and that soluble protein is obtained by the reversal of denaturation.

SH and S-S Groups.—The high cystine content of serum albumin facilitates an entirely different and independent test for the completeness of denaturation and the reality of reversal. In general native proteins have few, if any, free sulfhydryl and disulfide groups, while denatured proteins have a number of such groups corresponding to

the total number of cystine and cysteine groups in the protein (Mirsky and Anson, 1930*b*; and unpublished experiments). Serum albumin treated with acid acetone or trichloracetic acid has the number of free sulfhydryl and disulfide groups characteristic of a denatured protein, and when soluble serum albumin is produced by the reversal of denaturation there is a corresponding disappearance of these groups (unpublished experiments).

Historical

The reversal of the denaturation of serum albumin was probably observed by Michaelis and Rona (1910). The experiments were not conclusive and there was some confusion in their interpretation, and so this investigation unfortunately never received the attention it deserved. Spiegel-Adolf (1926), however, showed definitely that if heat coagulated serum albumin is dissolved in alkali or acid and then electrodialyzed, soluble, heat coagulable protein is again obtained. No attempt was made to crystallize the protein. Spiegel-Adolf believed, in addition, that certain compounds of serum albumin with acid and alkali can be heated without the protein being denatured at all. Actually, one cannot tell from the type of experiment given in support of this conclusion whether serum albumin has been denatured or not.

EXPERIMENTAL

Preparation of Native Serum Albumin.—Defibrinated horse blood is left in the cold until the corpuscles have settled. The serum is siphoned off and half saturated with ammonium sulfate which precipitates the globulins and the few corpuscles which remain suspended in the serum. To each liter of filtrate is added 20 gm. solid ammonium sulfate which results in an immediate and complete precipitation of the serum albumin in the amorphous form. Crystallization which is much slower and less complete than amorphous precipitation has the disadvantage that the protein may be fractionated and may in any case be changed on standing. The amorphous precipitate is filtered off and dialyzed in the cold against distilled water.

Preparation of the Acid Acetone Powder.—The preparation is the same as that of the acid acetone powder of globin (Anson and Mirsky, 1930). To a 5 per cent solution of serum albumin in 0.05 N HCl is added ten times its volume of acetone containing 2 cc. of 5 N HCl per liter. The resulting precipitate is filtered, washed with acetone, pressed as dry as possible, and then dried in the air. The dry albu-

min hydrochloride readily dissolves in water to give a clear solution. Most of the pigment present with the native serum albumin remains in the acid acetone when the protein is precipitated. The small amount of pigment which remains with the protein is more green and less yellow than the original pigment.

Neutralization Experiments.—If 0.1 N NaOH is gradually added to a 2 per cent solution of the acetone powder a point is reached at which a small precipitate is produced which increases with time. If 10 per cent more alkali is added the precipitate is redissolved. If 15 per cent less alkali is added no precipitate at all is formed.

The addition of an equal volume of ammonium sulfate to a solution which has been neutralized to the precipitation point causes further precipitation. Some 70 to 75 per cent of the protein, however, remains in the solution and has the properties of native serum albumin. The same result is obtained when the protein is allowed to stand half an hour in more acid or alkaline solution before being brought to the precipitation point and half saturated with ammonium sulfate. The table gives the results of such an experiment.

TABLE

Ml. of 0.1 N NaOH added to 10 ml. of 2 per cent acid albumin	Per cent of protein not precipitated by half saturation with ammonium sulfate
2.6 – precipitation point	74
0.8×2.6	77
0.9×2.6	73
1.1×2.6	68
1.2×2.6	78

Estimation of Yield with the Phenol Reagent.—To 1 ml. of filtrate from the solution half saturated with ammonium sulfate (in a Pyrex test tube, not a pointed centrifuge tube) is added 9 ml. of water, and after mixing, 2 ml. of 20 per cent trichloracetic acid. The suspension is centrifuged and the supernatant solution poured off. The precipitate is dissolved in 2 ml. of 0.1 N NaOH and transferred with 23 ml. of water to a 50 ml. Erlenmeyer flask. 1 ml. of the phenol reagent (Folin and Ciocalteau, 1927) is added and then 1 ml. of 3 N NaOH. After 10 minutes the blue color developed is compared with the blue color developed from a known amount of acid acetone albumin similarly precipitated with trichloracetic acid.

The estimation of proteins with the original phenol reagent of Folin and Denis (1912) as introduced by Wu (1922) does not yield colors proportional to the protein concentrations. Proportionality is obtained by the use of a higher concentration of the more soluble form of the phenol reagent described by Folin and Ciocalteau. Greenberg (1929) has made this same modification of Wu's procedure. The quantities he uses are somewhat different from ours and he adds the

phenol reagent after the alkali. In our experience, the procedure of Greenberg does not always yield perfectly clear solutions.

Effect of Salt.—If 1 ml. of saturated ammonium sulfate is added to the 10 ml. of the 2 per cent acid albumin, then on neutralization with 2.6 ml. of 0.1 N NaOH and half saturation with ammonium sulfate, 68 per cent of the protein remains in solution.

Effect of Heating.—The acid acetone solution is heated to 100°C. for 3 minutes. On neutralization a 70 per cent yield of soluble protein is obtained.

Effect of Trichloracetic Acid.—To 10 ml. of 2 per cent acid albumin is added 20 ml. of water and 4.5 ml. of 20 per cent trichloracetic acid. The precipitate is centrifuged, dissolved by the addition of a minimum amount of 0.1 N NaOH, and the solution after being made up to 12.6 ml. with water is half saturated with ammonium sulfate. The yield of soluble protein is 76 per cent.

In a similar experiment, native serum albumin is precipitated with 5 per cent trichloracetic acid and the precipitate dissolved in an amount of alkali exactly equivalent to the trichloracetic acid which remains with the protein. The yield of soluble protein is 74 per cent.

Reversal of Heat Denaturation.—A mixture of 10 ml. of 2 per cent dialyzed native serum albumin and 3 ml. of 0.1 N HCl is heated for 3 minutes at 100°C. and then while still hot neutralized with 2.5 ml. of 0.1 N NaOH. The resulting suspension is centrifuged. The clear supernatant liquid (which gives only a haze with trichloracetic acid) is rejected and the precipitate is readily dissolved in 3 ml. of 0.1 N HCl plus enough water to give a final volume of 15 ml. 1 ml. is removed to provide a standard in estimating the yield. The 14 ml. are neutralized with 2.9 ml. of 0.1 N NaOH and half saturated with ammonium sulfate. 65 per cent of the protein is left in the solution.

Crystallization of "Reversed" Serum Albumin.—The serum albumin may be crystallized from the neutralized solution of 2 per cent albumin by adding to each 10 ml. of the solution half saturated with ammonium sulfate 2 to 2.5 ml. of saturated ammonium sulfate. It is better, however, to start with a more concentrated solution such as can be obtained by neutralizing a 5 per cent solution of acid albumin with 0.2 N NaOH. In this case 1.5 to 2 ml. of saturated ammonium sulfate per 10 ml. of half saturated suffices. The soluble serum albumin can be crystallized completely which indicates that half saturation with ammonium sulfate results in complete precipitation of denatured serum albumin even in the presence of a great excess of native serum albumin. The crystals of "reversed" serum albumin appear to have the same form as those of normal, native serum albumin.

CONCLUSIONS

1. It is possible to prepare crystalline, soluble, heat-coagulable serum albumin from coagulated serum albumin.

2. In the cases so far studied, the more soluble a denatured protein, the more easily its denaturation can be reversed.

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THE PRODUCTION AND INHIBITION OF ACTION CURRENTS BY ALCOHOL

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This paper describes an investigation growing out of preliminary experiments begun in 1922 by Mr. E. S. Harris and the senior author.[1] It deals with lowering of P.D. by virtue of which alcohol is able to start and stop action currents in *Nitella*.

It has already been stated that chloroform and KCl can stimulate by lowering the P.D. at a given spot until the inflow of current from neighboring regions is sufficient to start a negative variation.

Ethyl alcohol can act in the same manner. An interesting example of this appears in Fig. 1.[2] The alcohol was applied near the center of the cell, as shown in Fig. 2, at the spot marked *B*. At the start the P.D. of *B* with reference to *D*, as shown by curve *B*, was about 100 millivolts positive, owing to the fact that *D* had previously been killed by chloroform.[1,3] When alcohol (1.5 M) was applied the P.D. gradually fell toward zero (as shown by the rise of the curve) and when it reached 80 millivolts an action current started as shown by the sudden jump of the curve. The excitation spread in both directions, as shown by the movements of curves *A* and *C* about a second later. At *A* and *C* recovery was normal but at *B* it was very slow. At the point marked 2 on the record the cell was stimulated electrically at *X*. A normal

[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673.

[2] This record was chosen for reproduction from many which were similar. The statements made about it apply qualitatively to all the others, and are in good agreement quantitatively.

[3] The experiments were performed on *Nitella flexilis* with the technique previously described (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 167, 355; Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930–31, **14**, 385). The method of measurement is essentially electrostatic. The temperature was very close to 20°C.

response is seen at A but only a very slight one at B: since the impulse does not pass on to C it is evident that the alcohol at B is acting as a block.

At the point marked 3 the shutter of the camera was closed and the motion of the photographic paper was stopped for 1 minute during

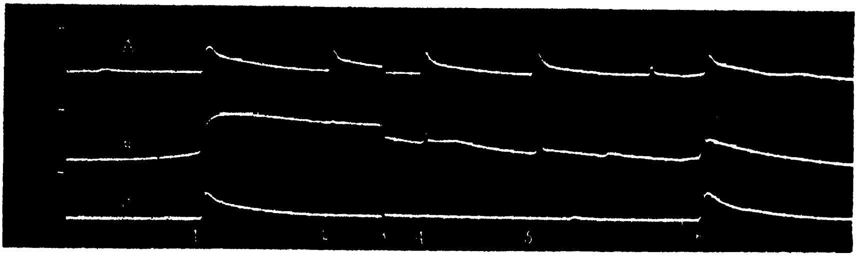

FIG. 1. Photographic record (employing a galvanometer with three strings) of an experiment arranged as in Fig. 2: 0.001 M KCl at all points except at B where 0.001 M KCl containing 1 5 M ethyl alcohol is applied. Curves A, B, and C show the P.D. of A, B, and C with reference to D (which has been killed by chloroform before starting the experiment). The intervals between time marks represent 5 seconds.

The effect of alcohol on starting and stopping action currents is seen in Curve B (for description see page 243).

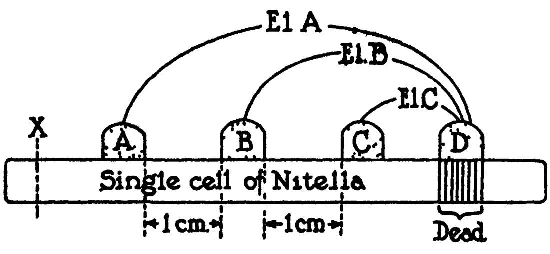

FIG. 2. Diagram to show the arrangement of an experiment: D is killed by the application of chloroform before the start of the experiment.

The cell is supported on a paraffin block, and is surrounded with air except where the contacts are applied.

which the alcohol was washed away by a stream of 0.001 M KCl. Cotton soaked in 0.001 M KCl was then replaced exactly on B: the record was set in motion, the camera was opened and at 4 the cell was stimulated electrically at X, producing a normal response at A, an

incomplete response at *B*, and none at *C*. Another stimulus at 5 had much the same result save that the response at *B* was greater. But the next stimulus (starting at *D*) at the spot marked 6 produced a much greater response: it will be noted that recovery has now made good progress and we see that the blocking effect of the alcohol has disappeared and that *A* now responds normally.

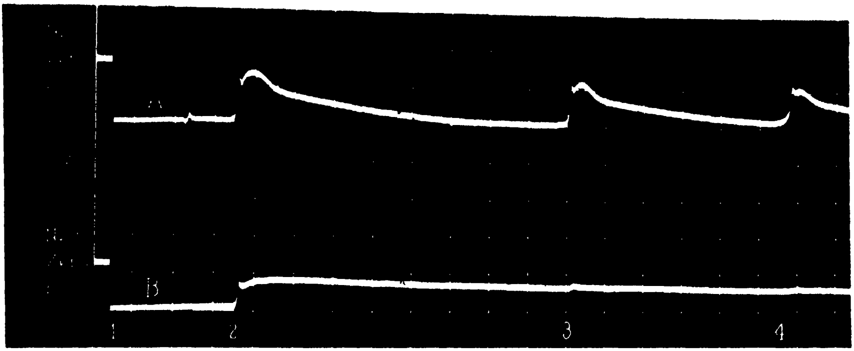

FIG. 3. Photographic record of an experiment arranged as in Fig. 2 with *C* omitted. At *A* and *D* 0.001 M KCl; at *B* 0.001 M KCl containing molar ethyl alcohol Curves *A* and *B* show the P.D. of *A* and *B* with reference to *D* (which has been killed by chloroform before the start of the experiment). The intervals between time marks represent 5 seconds.

Curve *B* shows that in this case alcohol had no apparent effect until an action current arrived from another part of the cell (for description see below).

In many cases there was a larger degree of recovery at *B* after passage of an action current, and the cell could again be stimulated within 30 seconds. In such cases the recovery was less with each successive response, because of the loss of P.D., but the curve rose to practically the same level each time.

It sometimes happens that the application of alcohol produces no apparent effect until the cell is stimulated. This is illustrated by Fig. 3, the experiment being arranged as in Fig. 2 with *C* omitted. At the start *B* was 65 millivolts positive to *D* and this was not altered by the application of molar alcohol at the point marked 1. At the point marked 2 the cell was stimulated electrically at *X* and a normal response occurred at *A*. A response is also seen at *B* (where alcohol is applied) but in this case there is practically no recovery. The subsequent stimuli at 3 and 4 produced only slight responses at *B*.

As is to be expected, the effect of alcohol varies with the concentration. With 0.5 M alcohol there was no lowering of P.D. and irritability was normal. With molar alcohol, the P.D. slowly fell, the loss not exceeding 20 per cent in 5 minutes when there were no action currents. An action current arriving from another part of the cell sent the P.D. approximately to zero, and recovery of P.D. was slow and incomplete. After several action currents, recovery was absent and propagated variations were no longer transmitted, but a response at the spot in contact with alcohol could still be secured by cutting another part of the cell.[4] On removal of alcohol, and washing, the P.D. returned completely in about 5 minutes, irritability, responses, and transmission becoming normal.

The application of 1.5 M alcohol produced results like those shown in Fig. 1, the loss of P.D. taking place during from 5 to 100 seconds after the application of alcohol. During the loss of P.D. stimulation usually occurred after which the P.D. approached zero. There was little or no recovery and electrical stimulation at such a point as X (Fig. 1) produced no response at B but cutting at X produced a death wave at B. When the alcohol was removed complete irritability returned in from 3 to 15 minutes.

Alcohol at a concentration of 2 M stimulated in a few seconds: the P.D. fell to and remained at zero, irritability and transmission were abolished but a response at B (Fig. 2) could be secured by cutting at X. As in the other cases the action of alcohol was hastened by an action current arriving from another part of the cell. On removing the alcohol no recovery was observed during 10 minutes, but after 20 minutes the potential had risen to approximately the original value and irritability had returned.

The effects of 3 M alcohol were much like those of 2 M, except that after the removal of alcohol and washing the cell, there was only par-

[4] Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930–31, **14**, 473. The response consisted in a rise of the curve a little above zero and a gradual falling to zero. The stimulus of the cut causes the inner surface to lose its P.D. first, thus causing the curve to go above zero. This may be similar to the effect of cutting when the cell is in contact with 0.01 M KCl, as explained in former papers (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 167) except that in the case of alcohol plus 0.001 M KCl the P.D.'s of each layer may be small.

tial recovery of potential, not to exceed 30 per cent, and no recovery of irritability. Usually the cell died within an hour or two.

The lowering of P.D. has been observed by Jost[5] in *Chara* and by Umrath[6] in *Nitella*.

In chemical stimulation repeated action currents are commonly observed[7] and it has been stated that this appears to depend on a rather sharp boundary of the stimulating agent which results in a rather steep electrical gradient in the neighborhood of the boundary. Unless this can be obtained we should not expect repeated action currents. In the case of alcohol a sharp boundary would not be expected since where alcohol and water meet a rather violent mixing occurs. This applies to a lesser extent when solutions of alcohol in water come in contact with water in the film covering the cell wall. It is therefore not surprising that we do not observe repeated action currents in our experiments.

It is most interesting to find that a non-electrolyte can reversibly alter P.D. to such an extent and it is important to discover how it is done. No doubt structural changes could account for it, *e.g.* by reversible coagulation or the formation of openings (as elsewhere discussed[1]), by changes in surface tension. On the other hand, alteration of the non-aqueous surfaces involving changes in the mobility of ions might bring it about. Alcohol might tend to dissolve out some of the non-aqueous constituents and thus change mobilities: it is probably more polar than the surface layers of the protoplasm and would therefore tend to increase their conductivity and the solubility of electrolytes in them.[8] Such changes might affect the inner and outer surfaces unequally, since these surfaces appear to differ. Further experiments will be undertaken to clear up some of these points.

Similar experiments on the sciatic nerve of the leopard frog (April)

[5] Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch.*, Abt. B, 1927, Abhandl. 13, Nov.

[6] Umrath, K., *Protoplasma*, 1930, **9**, 576.

[7] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 459.

[8] Merely increasing the conductivity without changing relative mobilities would reduce the resistance of the protoplasm without changing the P.D., but the observed value of the P.D. might be slightly raised for reasons given elsewhere (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1929–30, **12**, 761).

gave negative results. The nerve was exposed near its origin by opening the dorsal body wall in the lumbar region: in this way injury to the nerve could be easily avoided. In some cases the nerve was exposed just above the knee. Concentrations of ethyl alcohol ranging from 1 to 5 molar (in Ringer's solution) were then applied without causing the leg muscles to twitch although subsequent electrical stimulation showed that the nerve was functioning normally.

This result does not seem to be due to the fact that the alcohol does not penetrate to the nerve fibres, for alcohol (3.5 M) applied to the nerve established a complete block in about 4 minutes without causing an action current. It is quite possible that the absence of action currents is due to the absence of a sharp boundary, as already suggested for *Nitella*.

Concentrations up to 5 M applied to the intact skin did not cause the characteristic wiping reaction which was, however, promptly elicited by acetic acid.

On the other hand it must be remembered that one can taste 1.5 M ethyl alcohol without difficulty.

Kemp and Waller[9] state that 5 to 10 per cent alcohol in saline gives a temporary contracture of the frog's sartorius when the muscle is immersed in the solution. This is evidently somewhat different from the negative variation observed in *Nitella*.

SUMMARY

Suitable concentrations of ethyl alcohol (1 to 1.5 M) applied to a spot on a cell of *Nitella* lower the P.D. enough to cause action currents. The alcohol then suppresses action currents arriving from other parts of the cell and acts as a block. After the alcohol is removed the normal P.D. and irritability return.

Similar experiments on the sciatic nerve and skin of the frog produced only a negative result.

[9] Kemp, H. P., and Waller, A. D., *J. Physiol.*, 1908, **37**, xliii (Proc.).

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CRYSTALLINE PEPSIN

III. PREPARATION OF ACTIVE CRYSTALLINE PEPSIN FROM INACTIVE DENATURED PEPSIN

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INTRODUCTION

The results (1) of the experiments with crystalline pepsin isolated from crude pepsin preparations indicated that the material is a pure substance and that the proteolytic activity is a property of the protein molecule itself and is not due to the presence of a separate non-protein impurity. No indication of the presence of a more highly active non-protein molecule was obtained in the solubility measurements, inactivation experiments, or measurements of the rate of diffusion. In a sense however, this evidence is all negative in that it merely fails to show the presence of a more active molecule. It was shown, for instance, that the loss in activity in alkaline solution was quantitatively paralleled by the production of insoluble denatured protein. It could be objected, however, that the enzyme was liberated from the protein when the latter became denatured and that the enzyme itself was too unstable to exist alone. If it could be shown, however, that reversal of denaturation of the protein was accompanied by reactivation of the enzyme and that the native protein obtained in this way had the same activity as the original protein, strong proof would be furnished for the assumption that the activity was really a property of the protein molecule. In order to account for this result on the hypothesis that the activity was due to the presence of a small amount of a more highly active substance it would be necessary to suppose that the conditions for transforming the denatured protein into the original pro-

tein and for reactivating the enzyme were nearly identical—an assumption which is highly improbable.

It was found by Pawlow and Parastschuk (2) that pepsin solutions which had been inactivated by alkali recovered some of their activity when allowed to stand in nearly neutral solution. It was shown by the writer (1) that this alkali-inactivation of the enzyme is quantitatively paralleled by the formation of denatured protein as determined by the production of protein insoluble near the isoelectric point. The procedure used by Pawlow and Parastschuk for reactivating the enzyme was identical with that found by Anson and Mirsky (3) for the reversal of denaturation of proteins. This suggests that the reactivation of pepsin is due to the reversal of coagulation of the pepsin protein. If this explanation is correct it should be possible to isolate active pepsin protein from such reactivated solutions. The experiments reported in this paper show that this can be done.

Experimental Results

Pawlow and Parastschuk's results were repeated more carefully by Tichomirow (4) who apparently could recover about 10 per cent of the original activity using dog gastric juice. Tichomirow inactivated the enzyme by adding strong alkali or sodium carbonate and then titrated the solution to slightly less than neutrality as shown by litmus. When the solution was allowed to stand under these conditions a gradual increase in activity was found. Exact repetition of these experiments is difficult since at the time of Tichomirow's work pH measurements were not known. The exact degree of neutralization could not be duplicated with certainty from the data given. It is evident from Tichomirow's work, however, that the reaction is dependent upon the pH at which inactivation takes place, the pH and temperature at which reactivation occurs, and the length of time for which the solutions are allowed to stand under these conditions. In addition, the salt concentration and enzyme concentration are other possible variables.

It has been shown by Goulding, Borsook and Wasteneys (5) that two reactions are involved in the inactivation of pepsin by alkali. The first reaction is instantaneous and its *extent* is determined by the pH to which the solution is brought, while the *rate* of the second reaction

is determined by the pH. When the percentage inactivation caused by the instantaneous reaction is plotted against the pH a titration curve is obtained with a pK of about 6.9. This result has been confirmed with crystalline pepsin solutions, and it was shown in addition that the percentage inactivation was exactly parallel with the percentage of the protein denatured.

Preliminary experiments showed that a solution of crystalline pepsin which has been inactivated by titrating with NaOH to pH 10 or 11 (pink to nile blue) becomes slightly active again when allowed to stand

TABLE I

Effect of pH of Solution during Reactivation

100 ml. crystalline pepsin solution titrated to pH 10.5 with NaOH at 22°C. for 10 min. Titrated to pH 6.0 + HCl and diluted 1/8 with M/100 citrate buffer of different pH. Allowed to stand at 22° C. for 18 hrs. and activity determined.

pH of solution after diluting with citrate buffer		5.0	5.3	5.6	5.9	6.2
$[PU]_{ml.} \times 10^{3}$ of original solution	gelatin V	1.0	1.0	1.0	1.0	1.0
	casein F	100	100	100	100	100
After inactivation	gelatin V	0	0	0	0	0
	casein F	0	0	0	0	0
After reactivation	gelatin V	0.02	0.07	0.13	0.16	0.12
	casein F	0.10	0.40	0.40	0.20	0.10
Per cent of original, reactivated	gelatin V	2.0	7.0	13.0	16.0	12.0
	casein F	0.10	0.40	0.40	0.20	0.10

after partial neutralization. The yields, however, were less than 1 per cent instead of 10 per cent as described by Tichomirow. The conditions were therefore varied systematically in an attempt to increase the yield. The first condition studied was the effect of varying the pH to which the solution was brought after complete inactivation by alkali. It was found that the maximum reactivation was obtained at about pH 5.4 for the casein-hydrolyzing activity, while the gelatin liquefying activity was recovered more completely

in slightly more alkaline solution.[1] The results of one of these experiments are shown in Table I.

In the experiment just described inactivation was caused by strong alkali and was therefore probably due almost entirely to the instantaneous reaction rather than to the slower secondary reaction. It seemed possible that better yields might be obtained if the inactivation were brought about by the slow reaction. However, experiment showed that this was not the case but that on the contrary no reactivation could be obtained in solutions which had been allowed to inactivate slowly at lower pH's. The results of this experiment are shown in Table II.

TABLE II

Effect of pH during Inactivation on Reactivation

Series of 10 ml. crystalline pepsin solutions titrated to pH noted. Kept at 20°C. until more than 90 per cent of activity had been lost. Titrated to pH 5.4, kept at 22°C., and activity determined after 24 to 48 hrs.

pH at which inactivation occurred		7.0	8.0	8.5
$[PU]_{ml.} \times 10^3$ of original solution	gelatin V	4.0	4.0	4.0
	casein F	400	400	400
After inactivation	gelatin V	0.4	0.5	0.1
	casein F	0.10	0	0
After reactivation	gelatin V	0.4	0.6	0.2
	casein F	0.8	0.4	0.35
Per cent reactivated	gelatin V	0	2.5	2.5
	casein F	0	0.1	0.1

Effect of the Time of Standing after Neutralization.—When the solution is brought back to pH 5.4 and tested at once for activity it is found to be completely inactive. The activity slowly increases for

[1] The variation in the activity as measured by the liquefaction of gelatin or the hydrolysis of casein after alkali inactivation is due to the fact that the crude pepsin contains a gelatin liquefying enzyme which can only be removed by repeated crystallization. This gelatin splitting enzyme is more resistant to alkali than the pure pepsin. This point will be discussed more fully in a subsequent paper.

24 to 48 hours and then decreases so that apparently there are again two reactions, one of which leads to the formation of active enzymes while on the other hand, the active material is being slowly destroyed. The amount of active material actually recovered then is the result of these two reactions. The results of such an experiment are shown in Table III.

Effect of Concentration on Percentage of Reactivation.—The work of Anson and Mirsky on the reversal of denaturation of proteins indicates that the reaction is dependent somewhat upon the solubility of the denatured protein. Conditions which increase the solubility of the denatured protein are favorable for reversal while conditions which

TABLE III

Effect of Time at pH 5.4 on Reactivation

0.05 per cent pepsin solution titrated to pH 10.5 with NaOH at 22°C. for 10 min. Titrated to pH 5.4 with N/2 HCl. Activity determined after various time intervals at 22°C.

Hrs. at 22°C.	$[PU]_{ml.} \times 10^3$	
	Gelatin V.	Casein F.
0	0	0
2		0.02
18	0.03	0.20
42	0.03	0.21
65	0.04	0.25
90	0.045	0.12
114	0.040	0.10

cause precipitation of the denatured protein are unfavorable. It seemed possible therefore that better yields would be obtained with more dilute solutions and this was found to be the case. There is even some indication that the concentration of active material recovered is nearly constant so that the percentage of activity recovered increases with the dilution. Table IV shows the results of the experiment in which the solution was diluted to various extents after being titrated to pH 5.4. Practically, however, it is not possible to work with solutions containing much less than 1 mg. of nitrogen per ml. owing to the very large volume.

A number of other experiments were done in an attempt to increase the percentage yield but without success. It is quite possible that the difference between these results and those of Tichimorow are due to the difference between dog pepsin and pepsin from pigs, since Anson and Mirsky have found that there is very considerable difference in the ease of reversal of hemoglobin from different animals.

Tests for the Presence of Inhibiting Substances.—The possibility exists that the loss in activity is due not to destruction of the enzyme but to the formation of an inhibiting substance which depresses the activity of the enzyme and which disappears on standing at pH 5.4 and thus liberates the active enzyme. This possibility can be rendered remote by testing the effect of the inactivated solution upon the activity of normal pepsin solutions. If an inhibiting substance were present it would be expected that the activity of a known amount of active enzyme when added to this inactivated solution would be decreased since a large excess of inhibiting agent is usually necessary to completely inactivate the enzyme. No evidence for the presence of inhibiting substances could be found since active pepsin solutions diluted with a large excess of inactive solution show the same activity as when diluted with water. This result is shown in Table V.

TABLE IV

Effect of Concentration on Reactivation

Solution of crystalline pepsin titrated to pH 10.5 with N/2 NaOH at 20°C. for 10 min. Titrated to pH 5.4 with HCl, diluted with water, and kept at 22°C. for 18 hrs.

Mg. N/ml.		8	4	2	1	0.5
$[PU]_{ml.} \times 10^3$ (casein F.) —	original solution	800	400	200	100	50
	Immediately after titrating to pH 5.4	0	0	0	0	0
	After 24 hrs. at pH 5.4	1	1	0.7	0.5	0.4
	Per cent of activity recovered	0.1	0.25	0.35	0.5	0.8

Tests for Completeness of Inactivation.—As shown in the previous paper the denatured protein itself is rapidly digested by the enzyme, and this furnishes a very sensitive test for the presence of any active

pepsin in the solution after inactivation by alkali. The inactivated solution when brought to pH 2.5 to 3 forms a heavy precipitate of denatured protein. Under these conditions this protein is rapidly digested if any active enzyme is present. It was found that when the inactivated solution was titrated to pH 2.5 no increase in soluble nitrogen could be detected after 24 or 48 hours at 37°C. The addition of 4 per cent of the same solution which has been allowed to reactivate at pH 5.4 caused a marked increase in the amount of soluble nitrogen under these conditions (Table V). It may therefore be stated with

TABLE V

Activity Determination on Inactivated Pepsin Solution

6 per cent solution of crystalline pepsin titrated to pH 11.0 with N/2 NaOH

	$[PU]_{ml.} \times 10^{3}$	
	Gelatin V.	Casein F.
50 ml. titrated to pH 5 rapidly, slightly cloudy solution = a	0	0
100 ml. titrated to pH 3 rapidly, precipitate forms = b	0	0
25 ml. titrated to pH 5.4 and kept at 22°C. for 24 hrs. = c	0.05	0.5
Active pepsin solution diluted 1/100 with H_2O	1.5	150
with Solution a	1.6	165

0, 1 and 2 ml. Solution c added to 3 portions of 25 ml. of Solution b and nitrogen determined per ml. filtrate after 0 and 24 hrs. at 37°C.

Ml. reactivated Solution c	0	1	2
Mg. N/ml. filtrate after 0 hrs. at 37°C	0.10	0.12	0.15
after 24 hrs. at 37°C	0.10	0.20	0.25

considerable certainty that not more than 2 per cent of the activity present in the reactivated solution was present after inactivation due to incomplete destruction of the original activity. In this experiment also no filtration or separation was made so that complicating factors such as adsorption of the active material by the precipitate, etc. seemed to be ruled out.

It had been found before that a mixture of active and inactivated pepsin could be separated by precipitating the inactive and denatured protein with a mixture of sulfuric acid and sodium sulfate. In the

reactivated solutions obtained in the present work, however, it was found that the very small amount of active material present was carried down nearly completely by the denatured protein under these conditions, so that a new method of separation had to be worked out. After a number of failures it was found that most of the denatured protein could be precipitated from the solution by adding acid very slowly and carefully to the reactivated solution until it was just possible to filter off the precipitate formed. Under these conditions the active material is not carried down by the precipitate but remains in the filtrate. The addition of a slight excess of acid, however, causes all the active material to be retained by the precipitate. It seems probable that this result is due to the charge on the precipitate. The active pepsin is isoelectric at about pH 2.7 while the denatured protein is apparently isoelectric somewhere around 4.5, so that between these two pH's the two proteins are oppositely charged and precipitate each other, while if the reaction is adjusted so as to be on the alkaline side of the isoelectric points of both proteins they may be separated. The gelatin liquefying material is carried down with the precipitate even under these conditions so that this step serves to separate the reactivated pepsin from the "gelatinase" as well as from the denatured pepsin. The active pepsin may be recovered from this filtrate by adjusting to pH 3 and half saturating with magnesium sulfate. The precipitate obtained in this way contains about 2/3 of the total recovered activity but has a specific activity of only about 1/10 that of the original crystalline pepsin and evidently still contains large amounts of the denatured protein. Most of the latter may be removed by dialysis against 1/100 normal hydrochloric acid for 2 or 3 days. Under these conditions the denatured protein is partly digested by the active enzyme present. The resulting clear solution is precipitated with magnesium sulfate and the precipitate now has about half of its original specific activity. It still contains quite a large amount of some protein material which appears to be isoelectric at about pH 5 and from which the active enzyme can be separated only with great difficulty. Successive fractionation with magnesium sulfate at pH 5 gradually removes this inactive protein but no sharp separation could be made, and there is considerable loss in material at this point. Eventually, however, it was possible to obtain a small amount of a

TABLE VI

Inactivation and Reactivation of Pepsin and Isolation of the Reactivated Enzyme

Procedure	Fraction No.	Total [PU]		$[PU]_{gm.\ N}$	
		Gel. V.	Cas. F.	Gel. V.	Cas. F.
100 gm. crystalline pepsin dissolved in 1 l. H_2O		17	1700	1	100
Titrate to pH 10.5 (pink to nile blue) + N/2 NaOH (800 ml.) at 22°C. for 10 min.		<0.01	<1	0	0
Dilute to 10 l. Titrate to pH 5.4 with N/5 HCl, stirred in slowly so as to avoid local precipitation, at 22°C. for 24 hrs.	1	1.0	15	0.06	1.0
Titrate with N/5 HCl slowly until flocculent precipitate just forms (pH about 5.2). Filter. Filtrate	2	0.12	10		4
Filtrate 2. Titrate to pH 3.0 + H_2SO_4, $MgSO_4$ added till flocculent precipitate forms (about 350 gm. $MgSO_4$ per l.). Allowed to stand at 6°C. for 48 hrs. Supernatant siphoned off and precipitate concentrated by centrifuging. Centrifuged cake put in collodion sacs and dialyzed against 0.01 HCl at 22°C. for 48 to 60 hrs. Nearly clear solution. Centrifuge. Supernatant titrated to pH 5.4 with NaOH and $MgSO_4$ added until precipitate forms. Centrifuge, precipitate inactive. Supernatant	3	0.012	1	0.6	40
Supernatant 3. $MgSO_4$ added until flocculent precipitate forms. Centrifuge, precipitate weak activity. $MgSO_4$ added again to supernatant until precipitate forms. This process is continued until supernatant fluid has about proper specific activity per gm. N. The combined precipitates may be fractionated again and some active material recovered. *Combined Mother Liquors from $MgSO_4$ Precipitate.*	4	0.006	0.5	0.9–10	90–100
Solution 4. Titrate to pH 3.0 with H_2SO_4. Filter if cloudy and precipitate discarded. *Filtrate.* Saturate with $MgSO_4$ and filter. Precipitate	5	0.002	0.17	0.9–10	90–100
Precipitate 5 from 3 preparations combined. Dissolve at 40°C. with 0.2 to 0.3 ml. of water and 0.1 ml. N/10 Na acetate. Cool slowly. Typical hexagonal bipyramids in clusters form after 2 to 3 hrs.					

protein which had the general properties of the original crystalline pepsin. A summary of the method of isolation is shown in Table VI. About 2 kg. of the crystalline pepsin were used and the yields combined.

Control experiments like those shown in Table V were made on each preparation and showed that not more than 2 per cent of the activity could have been due to the presence of original enzyme which had not been inactivated. A small amount of this reactivated protein was crystallized under the same conditions as were used in crystallizing the original pepsin. It formed typical hexagonal bipyramids indistinguishable from those of the original pepsin. The activity as

TABLE VII

Comparison of Properties of Original and Reactivated Pepsin

	$[PU]_{gm. N}$			Mg. N to coagulate 5 ml. milk at 35°C. in 2 hrs.	$[\alpha]^{D}_{22°C.}$ pH 4.6
	Gelatin V.	Casein F.	Edestin F.		
Original crystalline pepsin	1.1	92	98	<0.001>0.0005	−70
Reactivated pepsin	1.0	95	100	<0.001>0.0005	−100 ± 10
	0.8	98	110		

measured by the liquefaction of gelatin, the hydrolysis of casein, the hydrolysis of edestin and the coagulation of milk was the same as that of the original pepsin.[2] The optical rotation was measured on a very dilute solution and apparently showed a slightly different value from that of the original pepsin. The reading was somewhat uncertain, however, and it is doubtful whether this difference is significant. The

[2] It will be noted that the activity of the recovered as well as the original pepsin as determined by the digestion of edestin is only half of that originally reported for crystalline pepsin. This difference is apparently due to some difference in the edestin preparation since a different preparation was used in the present work. Unfortunately the edestin preparation used previously had been entirely used up but a sample of the original pepsin preparation was at hand both dry and in glycerin solution. This preparation now shows the same activity in all other respects as that found earlier but is apparently less active with the new edestin.

protein is denatured and the activity lost again in alkaline solution. The results of these determinations are shown in Table VII.

Attempts were made to compare the solubility of the reactivated and original pepsin but owing to lack of material these were not conclusive. It was found that the active material forms solid solutions with the protein which is carried along in the purification and which is apparently isoelectric at about pH 5. Addition of this protein to pure pepsin caused a marked decrease in solubility. The results with the rennet action confirmed Pawlow and Parastschuk's conclusion that the apparent separation of rennet from casein by alkaline activation is really due to the fact that the pepsin is reactivated under the conditions used for the coagulation of milk but not under the conditions used for the digestion of casein.

Experimental Methods

The methods used were the same as those described in the study of crystalline pepsin. The pH determinations were made colorimetrically without correcting for any effect of the protein present and are only approximately correct.

SUMMARY

1. Pepsin solutions which have been completely denatured and inactivated by adjusting to pH 10.5 recover some of their activity when titrated to about pH 5.4 and allowed to stand at 22°C. for 24 to 48 hours.

2. Control experiments show that this inactivation and reactivation are probably not due to the effect of any inhibiting substance.

3. A method of isolation of the reactivated material has been worked out.

4. The reactivated material recovered in this way is a protein with the same general solubility, the same crystalline form, and the same specific proteolytic activity as the original crystalline pepsin.

5. This furnishes additional proof that the proteolytic activity is a property of the protein molecule.

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METHÄMOGLOBIN-ERZEUGUNG UND ATMUNGSSTEIGERUNG DURCH ORGANISCHE FARBSTOFFE

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PROBLEMSTELLUNG

Harrop und *Barron* haben gezeigt, daß kernlose rote Blutkörperchen bei Gegenwart von Methylenblau Zucker verbrennen, während ohne Methylenblau bekanntlich nur Glykolyse mit Milchsäure als Endprodukt auftritt. *Barron* zeigte die gleiche Wirkung von Methylenblau auf unbefruchtete Echinodermeneier, deren natürliche, aber geringfügige Atmung durch Methylenblau ebenfalls verstärkt wird. *Barron* zeigte ferner, daß auch andere organische Farbstoffe mehr oder weniger denselben Effekt haben. Der Grad der Wirksamkeit der verschiedenen Farbstoffe schien im wesentlichen von dem Bereich ihres Oxydationsreduktionspotentials bestimmt zu werden. Alle wirksamen Farbstoffe gehörten zu den reversibel reduzier- und oxydierbaren, und wenn man das Potential eines Gemisches gleicher Mengen der oxydierten und der reduzierten Form eines Farbstoffs als das Normalpotential des Farbstoffs bezeichnet, so kann man sagen, daß nach *Barron* für die Wirkung auf Seesterneier die folgende Regel erkennbar ist: Das Optimum der Wirkung zeigten Farbstoffe von einem Normalpotential von etwa 0 Volt (gegen die Normalwasserstoffelektrode) bei p_H 7, während Farbstoffe sowohl von positiverem wie von negativerem Normalpotential schlechter wirkten. Bei positiveren Farbstoffen, bei denen das Maximum der Wirkung schon überschritten war (den Indophenolen), konnte der Effekt durch Erhöhung der Konzentration verbessert werden, bei Farbstoffen von negativerem Normalpotential (den Indigosulfonaten, Safranin) war der Effekt stets geringer, und zwar entsprechend der Negativität des Normalpotentials. Die Wirkung genügend zahlreicher Farbstoffe auf die At-

mung kernloser roter Blutzellen ist dagegen noch nicht untersucht worden.

Die Deutung der Farbstoffwirkung wurde durch *Warburg* und fast gleichzeitig durch *Wendel* gefunden. Die Theorien dieser Autoren bringen die Wirkung mit der Bildung von Methämoglobin in Zusammenhang, unterscheiden sich aber in den Einzelheiten des Mechanismus. *Warburg*s Deutung ist durch umfangreiches Material so erhärtet, daß wir ihr den Vorzug geben, zumal da inzwischen *Wendel* selbst den wesentlichen Unterschied seiner Theorie von der *Warburg*schen zurückgenommen hat.

Hiernach erzeugt der Farbstoff aus dem Hämoglobin Methämoglobin, indem er das Ferroeisen des Hämoglobins zu Ferrieisen oxydiert. Der hierdurch reduzierte Farbstoff wird an der Luft wieder oxydiert. Das Methämoglobin wirkt unter Vermittlung der Struktur des lebenden Blutkörperchens als Oxydationsmittel für den Zucker und wird dadurch selbst zu Hämoglobin reduziert. Das Methämoglobin spielt die Rolle des Atmungsferments, bedarf aber der Mitwirkung des Farbstoffs, weil reduziertes Methämoglobin, d. h. Hämoglobin, durch Sauerstoff nicht spontan oxydiert wird (sondern stattdessen eine Anlagerungsverbindung mit Sauerstoff, das Oxyhämoglobin, bildet, in welchem das Eisen zweiwertig bleibt).

Ob auch für Echinodermeneier ein analoger Mechanismus gültig ist, ist noch nicht erwiesen aber durchaus möglich, da ein hämoglobinähnliches Pigment gerade in Echinodermeneiern nachgewiesen worden ist und überhaupt hämatinähnliche Substanzen in Zellen verschiedenster Art weit verbreitet sind.

Es ist die Aufgabe dieser Arbeit, die atmungssteigernde Wirkung von verschiedenen Farbstoffen mit ihrer Methämoglobin erzeugenden Eigenschaft zu vergleichen und zu untersuchen, ob diese Eigenschaft der Farbstoffe zu ihrem Normalpotential in Beziehung steht. Diesem eigentlichen Inhalt der Arbeit schicken wir eine Erörterung über die chemische Natur des Methämoglobins voraus.

Die Beziehungen von Oxyhämoglobin zu Methämoglobin

Die heute am besten gesicherten Anschauungen über die Beziehungen von Hämoglobin, Oxyhämoglobin und Methämoglobin sind folgende: Hämoglobin ist eine Ferroverbindung, Oxyhämoglobin ist

ein Sauerstoffadditionsprodukt derselben, welches das Eisen aber noch in der Ferroform enthält, und Methämoglobin ist ein wahres Oxydationsprodukt des Hämoglobins, welches Ferrieisen an Stelle von Ferroeisen aber keinen angelagerten Sauerstoff enthält.

Diese Auffassung ist wohl zum erstenmal in klarer Weise von *Conant* ausgesprochen worden. Er bezeichnet Oxyhämoglobin als ein Oxygenationsprodukt und Methämoglobin als ein Oxydationsprodukt des Hämoglobins. Wenn diese Anschauung richtig ist, so darf sich Hämoglobin vom Methämoglobin nur durch *ein* Oxydationsäquivalent unterscheiden, wie irgendeine Ferriverbindung von der zugehörigen Ferroverbindung, während Oxyhämoglobin von Hämoglobin sich um *vier* Oxydationsäquivalente unterscheidet, entsprechend der Tatsache, daß 1 Mol Hämoglobin 1 Mol O_2 bindet. Die Behauptung von *Conant*, daß Hämoglobin und Methämoglobin sich nur um *ein* Oxydationsäquivalent unterscheiden, ist von *Roaf* und *Smart*, *Quagliariello* und *Nicloux* angegriffen worden. Obwohl *Conant* selbst diesen Einwand durch einen späteren Versuch widerlegt hat, scheint es bei der Wichtigkeit der Sache wünschenswert, einen weiteren Beweis dafür zu erbringen. Dies konnte durch folgende Versuchsanordnung in sehr einfacher Weise geschehen.

Wir legen dem Versuch die Annahme zugrunde, daß 1 Mol Hämoglobin mit 1 Mol O_2 sich zu Oxyhämoglobin verbindet, und daß dieser ganze Sauerstoff in Freiheit gesetzt wird, wenn man Oxyhämoglobin mit einem Überschuß von Kaliumferricyanid behandelt. Wenn man nun Oxyhämoglobin mit einer zur vollen Oxydation nicht ausreichenden Menge von Kaliumferricyanid versetzt, so wird nur ein Teil des Oxyhämoglobins in Methämoglobin verwandelt, und zwar zeigt der Vergleich des entwickelten Sauerstoffs mit der angewandten Kaliumferricyanidmenge, daß 1 Mol Kaliumferricyanid 1 Mol O_2 in Freiheit setzt. Andererseits, wenn man eine bestimmte Menge von Oxyhämoglobin mit einem Überschuß von Kaliumferricyanid behandelt, die entwickelte Sauerstoffmenge mißt und in einer Versuchsreihe feststellt, wieweit man mit der Menge des Kaliumferricyanids heruntergehen kann, ohne die Menge des entwickelten Sauerstoffs zu verringern, so findet man, daß 1 Mol Hämoglobin mit *1 Mol* Kaliumferricyanid reagiert. Verringert man die Menge des Kaliumferricyanids unter diesen Schwellenwert, so wird genau der unserer Annahme

entsprechende Bruchteil von Sauerstoff entwickelt. Hieraus folgt eindeutig, daß das Methämoglobin sich nur um *ein* Oxydationsäquivalent von Hämoglobin unterscheidet.

Dieser Versuch zeigt, daß pro Mol Kaliumferricyanid 1 Mol O_2 frei wird.

Der Versuch zeigt, daß zur Bildung von Methämoglobin aus 1 Mol Oxyhämoglobin gerade *1 Mol* Kaliumferricyanid erforderlich ist.

VERSUCH I

Überschuß an Oxyhämoglobin

K_3FeCN_6 Millimol	O_2 theor.* cmm	O_2 erhalten cmm
0,002	44,8	44,0

* Berechnet unter der Annahme, daß 1 Mol Ferricyanid 1 Mol O_2 in Freiheit setzt.

VERSUCH II

Überschuß an Ferricyanid

K_3FeCN_6 Millimol	O_2 theor.** cmm	O_2 erhalten cmm
0,0076	86,1	85,2
0,0038	86,1	85
0,0019	43	41,2

** Berechnet unter der Annahme, das 1 Mol Ferricyanid mit 1 Mol Hämoglobin reagiert.

Hiermit ist allerdings nicht bewiesen, daß alles, was man nach seinem spektroskopischen Verhalten Methämoglobin nennt, identisch ist mit dem durch Kaliumferricyanid erzeugten Methämoglobin; jedoch stehen alle Angaben, daß es verschiedene Substanzen gäbe, die man nach dem spektroskopischen Verhalten als Methämoglobin bezeichnen müßte, auf schwachen Füßen.

Die Behauptung, daß eine charakteristisch gelegene Bande, welche überdies die Eigenschaft hat, durch Zusatz von Natriumfluorid bei p_H 6 in typischer Weise verschoben zu werden (s. hierüber den nächsten Abschnitt), verschiedenen chemischen Individuen zukomme, be-

dürfte eines sehr eindeutigen Beweises. So sei z. B. auch erwähnt, daß nach dem *Warburg*schen Vorschlag durch Amylnitrit erzeugtes Methämoglobin dieselbe Bande und dieselbe Verschiebung dieser Bande durch Natriumfluorid zeigt wie das durch Kaliumferricyanid erzeugte. Nur kommt erschwerend für die Beurteilung der Reaktion mit Amylnitrit der Umstand hinzu, daß bei protrahierter Einwirkung von Amylnitrit das Methämoglobin wieder verschwindet und Hämatin dafür entsteht.

Methodik

Die Atmungsversuche wurden mit dem *Barcroft*-Manometer in der Anordnung des *Warburg*schen Mikrorespirationsapparats ausgeführt. Die Technik ist dieselbe wie in der früheren Arbeit.

Die Versuche über die Erzeugung von Methämoglobin aus gelöstem Oxyhämoglobin wurden teils spektroskopisch, teils gasanalytisch ausgeführt. Der spektroskopische Nachweis ist durch die Eigenfarbe der anzuwendenden Farbstoffe erschwert, aber bei geeigneten Farbstoffen, wie Methylenblau, folgendermaßen leicht zu erbringen: Die Mischung von gelöstem Blutfarbstoff und Methylenblau wird nach genügender Einwirkungszeit, z. B. 5 Minuten, mit etwas Kaliumjodid in Substanz versetzt. Dabei fällt das Methylenblau fast quantitativ als Jodid aus. Die in Lösung bleibende Spur ist so gering, daß sie die spektroskopische Analyse des für Methämoglobin wichtigen Spektralbereiches nicht stört. Von den beiden Absorptionsbanden des Methylenblaus stört an sich überhaupt nur die kurzwelligere, und diese ist unter diesen Umständen in den angewandten Schichtdicken nicht mehr bemerkbar. Bei reichlicher Menge von Methämoglobin sieht man die Bande um 638 $\mu\mu$ direkt. Um sie sicher zu identifizieren, wurde nach *Ville* und *Demien* und *Moritessier* folgendes Verfahren angewandt: Die Lösung wurde durch Zusatz von primärem Kaliumphosphat in Substanz angesäuert (p_H etwa 6) und mit reichlich Natriumfluorid in Substanz versetzt. Hierdurch wird der Methämoglobinstreifen erstens nach dem kurzwelligen Ende verschoben (Mitte der Bande um 610$\mu\mu$), zweitens wird die Bande schmaler und intensiver und der Nachweis dadurch empfindlicher. Jedenfalls ist die richtige Verschiebung der Bande durch Fluor ein eindeutiges Kriterium für das Vorhandensein von Methämoglobin. Noch einfacher

gestaltet sich der Nachweis von Methämoglobin bei Anwendung von solchen Farbstoffen, die keine störenden Absorptionsbande aufweisen. Versetzt man eine Lösung von Oxyhämoglobin mit Phenolindophenol bei p_H etwa 6, so tritt unmittelbar die braune Farbe des Methämoglobins auf. Die spektroskopische Untersuchung zeigt die typische, durch Natriumfluorid verschiebliche Methämoglobinbande; das Spektrum des Phenolindophenols stört in diesem Falle nicht.

Die gasanalytische Methode zum Nachweis des Methämoglobins beruht darauf, daß bei der Umwandlung von Oxyhämoglobin in Methämoglobin Sauerstoff frei wird und eine Druckerhöhung im Manometer zu beobachten ist.

Die Umwandlung des Oxyhämoglobins durch ein Oxydationsmittel besteht in der Oxydation des Hämoglobins zu Methämoglobin unter gleichzeitiger Reduktion des Oxydationsmittels. Dieser Vorgang erzeugt keine Änderung des Gasdruckes. Aber gleichzeitig wird der Sauerstoff des Oxyhämoglobins dabei in Freiheit gesetzt. Ist das Reduktionsprodukt des angewandten Oxydationsmittels nicht autoxydabel, so tritt pro Mol erzeugtes Methämoglobin 1 Mol Sauerstoffgas auf. Dies ist der Fall, wenn Ferricyankalium als Oxydationsmittel angewendet wird, wie *Haldane* gezeigt hat. Ist aber das Reduktionsprodukt des angewendeten Oxydationsmittels autoxydabel, so verbraucht es bei der Reoxydation einen Teil des frei gemachten Sauerstoffs. In quantitativer Hinsicht sollte man folgendes erwarten: Die Oxydation von 1 Mol Hämoglobin (einer Ferroverbindung) zu Methämoglobin (der zugehörigen Ferriverbindung) erfordert $\frac{1}{2}$ Mol Methylenblau und dieses, wenn reduziert, verbraucht $\frac{1}{4}$ Mol Sauerstoff zur Reoxydation. Es sollten also, wenn ein organischer Farbstoff benutzt wird, drei Viertel derjenigen Sauerstoffmenge frei werden, welche durch Ferricyankalium frei gemacht werden kann, wenn alles Hämoglobin in Methämoglobin verwandelt wird. Dies gilt wenigstens für den Fall, daß ein Überschuß des organischen Farbstoffs angewendet wird, so daß jedes Molekül Farbstoff nur einmal als Oxydationsmittel auf Hämoglobin wirkt und, wenn es danach durch Sauerstoff reoxydiert wird, kein Hämoglobin mehr zum weiteren Angriff vorfindet. Bezeichnen wir die durch Ferricyankalium entwickelte Menge Sauerstoff als die maximale, so sollte also im Falle der organischen Farbstoffe drei Viertel der maximalen Menge Sauerstoff frei werden.

Der Versuch zeigt nun, daß im allgemeinen weniger als drei Viertel der maximalen Sauerstoffmenge entwickelt wird. Nur bei einigen Indophenolen wird mit guter Annäherung dieser Wert oft erreicht. Hier kommt es sogar gelegentlich vor, daß er ein wenig überschritten wird. Ein solches Vorkommnis ist verständlich, wenn man annimmt, daß hin und wieder ein Molekül des Farbstoffs mehrfach oxydiert und wieder reduziert wird. Bei Anwendung von Methylenblau entsteht gewöhnlich etwa die Hälfte (in nicht ganz reproduzierbarer Weise), mit Indigosulfonaten etwa ein Fünftel, mit Safranin oder Rosindulin nur ein winziger Bruchteil der maximalen Menge Sauerstoff oder gar nichts.

Die Ursache ist, daß bei Anwendung selbst der wirksamsten Farbstoffe als Oxydationsmittel nur ein Teil des Hämoglobins in Methämoglobin verwandelt wird; der Rest bleibt Oxyhämoglobin.

Die Geschwindigkeit der Methämoglobinbildung, gemessen an der Geschwindigkeit der Sauerstoffentwicklung, ist bei den Farbstoffen auf alle Fälle viel kleiner als bei der Anwendung von Ferricyankalium, wo die Reaktion mit unmeßbar großer Geschwindigkeit verläuft, d. h. so schnell, daß die Einstellungszeit des Manometers zum Gleichgewicht größer ist als die Zeitdauer der chemischen Reaktion. Bei den Farbstoffen dagegen ist die chemische Reaktion so langsam, daß man sie am Manometer leicht verfolgen kann. Bei einem solchen Versuch zeigt sich nun, daß die Sauerstoffentwicklung nach der Vermischung von Farbstoff mit Hämoglobin mit relativ großer Geschwindigkeit einsetzt, aber dann langsamer wird und schließlich stehen bleibt, ohne daß der theoretisch erwartete Endwert erreicht wird. (Außer, wie erwähnt, bisweilen bei den Indophenolen.) Die spektroskopische Beobachtung zeigt in einem solchen Falle, daß nur ein Teil des Hämoglobins in Methämoglobin verwandelt ist.

Man könnte den Eindruck gewinnen, als ob die Reaktion zu einem Gleichgewicht führe. Ein wahres Gleichgewicht kann aber nicht vorhanden sein, denn das wahre Gleichgewicht eines Gemisches von Hämoglobin und Sauerstoff bei Gegenwart eines Oxydationskatalysators kann nur die vollständige Oxydation zu Methämoglobin sein. Man muß vielmehr annehmen, daß die oxydierende Fähigkeit der Farbstoffe für Hämoglobin mit der Zeit erlahmt. Es ist eine kinetisch, nicht eine thermodynamisch begründete Erlahmung. Als wahr-

scheinliche Erklärung hierfür soll angeführt werden, daß die Farbstoffe in einer Hämoglobinlösung nicht auf die Dauer in echter Lösung bleiben. Es bilden sich körnige, oft an den Gefäßen haftende, oder in der Lösung schwebende gefärbte Partikel, und so wird der Farbstoff als wirksames Agens allmählich ausgeschaltet und die Konzentration des echt gelösten Anteils des Farbstoffs wird so klein, daß die Geschwindigkeit der Reaktion praktisch gleich Null wird.

Unter diesen Umständen können wir die Fähigkeit der verschiedenen Farbstoffe zur Erzeugung von Methämoglobin beurteilen nach der Sauerstoffmenge, welche sie aus einer gegebenen Oxyhämoglobinlösung überhaupt in Freiheit zu setzen vermögen. Wir drücken diese Sauerstoffmenge am besten als Bruchteil derjenigen Sauerstoffmenge aus, welche in einem Parallelversuch mit der gleichen Oxyhämoglobinlösung und Ferricyankalium in Freiheit gesetzt wird. Die Oxyhämoglobinmengen wurden so gewählt, daß der Kontrollversuch mit einem Überschuß von Kaliumferricyanid etwa 100 cmm O_2 ergab. Die Zahlen der nachfolgenden Tabelle geben an, wieviel Prozent der im Parallelversuch mit Kaliumferricyanid entwickelten Sauerstoffmenge durch die verschiedenen organischen Farbstoffe in Freiheit gesetzt werden.

Farbstoff	O_2 in % der im Parallelversuch mit K_3FeCN_6 freigemachten O_2-Menge	Normalpotential bei p_H 6
Chlorphenol-Indophenol	85–105	+0,295
Phenol-Indophenol	etwa 83	+0,28
Gallocyanin	„ 40	+0,080
Methylenblau	60–70	+0,047
Azur B	etwa 66	Fast wie Methylenblau
Indigotetrasulfonat	„ 50	+0,006
Gallophenin	25–30	−0,077
Indigodisulfonat	etwa 20	−0,069
β-Anthrachinonsulfonat	16–20	−0,20
Safranin	etwa 5	etwa −0,2
Rosindulin	0	−0,221
Kristallviolett	0	Äußerst schwer und nicht reversibel reduzierbar
Methylorange	0	
Fuchsin	0	
Säurefuchsin	0	

Literatur für die Potentialwerte findet sich bei *L. Michaelis*, Oxydations- und Reduktionspotentiale und bei *R. Wurmser*, Oxydation et Réduction, S. 318. Die Mitteilung über Rosindulin ist im Druck.

Atmungssteigerung durch organische Farbstoffe

Die Versuche mit Methylenblau hatten gezeigt, daß der Grad der Atmungsbeschleunigung nur in mäßigem Maße von der Konzentration des Methylenblaus abhängt. Aus diesem Grunde konnten wir uns darauf beschränken, die Vergleichung der verschiedenen Farbstoffe in annähernd derselben Konzentration durchzuführen, und zwar bei einer endgültig 0,6 millimolaren Konzentration an Farbstoff. Farbstoffe, welche mit den gewöhnlichen Reduktionsmitteln überhaupt nicht oder jedenfalls nicht in reversibler Weise reduziert werden können, haben gar keinen Einfluß auf die Atmung. (Triphenylmethanfarbstoffe und Azofarbstoffe.) Reversible Farbstoffe von sehr negativem Potentialbereich, welche kein oder nur zweifelhaft Methämoglobin bilden, haben ebenfalls keinen Einfluß auf die Atmung. (Safranin, Rosindulin.) Farbstoffe von etwas positiverem Normalpotential beginnen eine geringe Wirkung zu zeigen, und zwar wird diese bei Indigotetrasulfonat eben bemerkbar, während sie bei dem etwas negativerem Indigodisulfonat noch nicht vorhanden ist. Die Wirkungen sind schwach und quantitativ nicht sehr gut reproduzierbar. Man kann deshalb hier keine zu hohen Ansprüche stellen an genaue Übereinstimmung der Reihenfolge des Potentials und der atmungssteigernden Wirkung, vielmehr sagt man besser, daß Farbstoffe vom Anthrachinonsulfonat bis zu den Indigodisulfonaten eine geringe, schlecht reproduzierbare oder gar keine Wirkung zeigen. Sobald man aber in das Potentialgebiet des Methylenblaus kommt, wird die Wirkung deutlich, erreicht bald ein Maximum und wird nun bei weiterer Potentialerhöhung nicht mehr überschritten. Methylenblau, Azur B, Gallocyanin und sogar die Indophenole bis hinauf zu den Farbstoffen mit den höchst erreichbaren Normalpotentialen haben im großen ganzen die gleiche Wirkung. Bei den Seesterneiern hat allerdings *Barron* beschrieben, daß Gallocyanin und die Indophenole schlechter als Methylenblau wirken; dieser Befund trifft jedoch für unser Beobachtungsmaterial nicht zu.

Die zunehmende Erhöhung des Potentials hat offenbar deshalb keinen weiteren Effekt, weil die Geschwindigkeit der Atmung bei genügender Positivität des Potentials durch irgendeinen anderen Faktor limitiert wird.

Aus den Versuchen erkennt man, daß in dem Maße, in dem die Farbstoffe positiver werden, ihre atmungsbeschleunigende Wirkung auftritt und mehr und mehr deutlich wird. Zwischen Indigodisulfonat und Indigotetrasulfonat liegt die Schwelle, bei der die Wirkung auf die Atmung deutlich wird. Bei weiterer Positivierung des Potentials erreicht sie dann bald ihren maximalen Wert, der nicht überschritten wird.

ZUSAMMENFASSUNG

1. Es wird bestätigt, daß 1 Mol Oxyhämoglobin nur mit *1 Mol* Ferricyanid reagiert, wenn es in Methämoglobin verwandelt und dabei der gesamte O_2 des Oxyhämoglobins in Freiheit gesetzt wird.

2. Organische Farbstoffe können als Oxydationsmittel auf Hämoglobin wirken, wobei sie es in Methämoglobin verwandeln. Diese Reaktion verläuft jedoch selbst bei Anwesenheit von überschüssigem Sauerstoff in der Regel nicht vollständig, sondern kommt, je nach dem Potentialbereich des Farbstoffs, früher oder später zum Stillstand. Dieser Stillstand ist kein thermodynamisches Gleichgewicht, sondern beruht auf einer kinetischen Hemmung.

3. Die Wirkung der Farbstoffe, die Atmung kernloser roter Blutzellen zu erhöhen, steigt in gleichem Sinne wie ihr Potentialbereich positiver wird und ihre Fähigkeit, Methämoglobin zu erzeugen, größer wird. Sie erreicht jedoch schon bei den Farbstoffen im Potentialbereich des Methylenblaus ihr Maximum, so daß jenseits dieses Bereiches eine Parallelität der beiden Wirkungen nicht mehr erkannt werden kann.

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THE NATURE OF THE RESPIRATORY SUPPLEMENT

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When the oxygen consumption of rabbit erythrocytes and that of saline extracts of rat livers (or various other organs), are measured separately, and compared with what happens in a mixture of both, the oxygen consumption is much higher in the mixture than the sum of the oxygen consumptions in the 2 separate systems. This has been shown by the authors previously;[1] the effect of the liver extract has been confirmed by Zeile and Euler.[2]

This paper presents the attempts to elucidate the nature of this reaction. For brevity's sake the active principle of the liver extract will be designated as the respiratory supplement.

1. The supplement deteriorates and may even be destroyed by various mechanical treatments of the liver extract. Extended shaking, in presence or in absence of oxygen damages the supplement. When the extract is centrifuged the supplement activity both of the sediment and of the supernatant fluid is diminished and by mixing the 2 fractions is not restored to its original strength.

2. Several attempts were made to isolate the supplement by adsorption. Sand, Kaolin, Kieselgur adsorb the supplement to only a small extent, according to the amount applied, but never completely. Colloidal iron hydroxyd adsorbed the supplement completely in several experiments, in which the adsorbent was used in an amount sufficient to remove the proteins. Colloidal aluminum hydroxyd removed it completely when applied in so small an amount as to remove only a small part of the proteins. No attempt to recover the supplement by elution with N/20 ammonia was successful.

3. Heating to 56° diminishes the supplement considerably though there is no critical temperature for this effect. The inactivation by heat seems to run parallel to the formation of a coarse turbidity or

[1] Michaelis, L., and Salomon, K., *J. Gen. Physiol.*, 1930, **13**, 683.

[2] Zeile, K., and v. Euler, H., *Hoppe-Seyler's Z. f. physiol. Chem.*, 1931, **195**, 35.

flocculation in the previously slight and homogeneous extract. Freezing did not impair the effect of the supplement at all.

4. The liver extract does not seem to be able to convert hemoglobin to methemoglobin, at least not to a degree detectable with certainty.

5. The supplementary effect of methylene blue (instead of liver extract) first observed by Harrop[3] and Barron[4] and investigated subsequently by Barron,[5] O. Warburg[6] and Wendel[7] is manifested only when the erythrocytes are intact and vanishes in experiments with hemolyzed erythrocytes. This is sometimes the case with liver supplement also as described in our previous paper. However, this phenomenon is not as regular with the liver supplement as with methylene blue. Sometimes the previous laking of the erythrocytes by repeated freezing and thawing, which destroys the effect of methylene blue, has only a slight damaging effect upon the action of the liver supplement.

It is, therefore, evident that the supplement is not simply comparable in nature and action with methylene blue. It has some intrinsic connection with what we may call the colloidal state, or a remainder of some kind of "structure," in the liver extract.

Press juice of liver obtained with Kleinmann's hydraulic micropress and diluted to a volume comparable to that of the usual extract, acts like the extract.

It is noteworthy, furthermore, that the sum of the oxygen consumption of the separated systems (the liver extract and the blood corpuscles) has no noticeable correlation with the oxygen consumption of the mixture. It may happen that the oxygen consumption of each of the components is negligibly small and the oxygen consumption in the mixture is very large.

The reproducibility of the original experiment with liver extract and intact erythrocytes, such as described in the previous paper turned out to be very good. Out of about 75 independent experiments there were only 3 in which the oxygen consumption of the mixture was not at least 3 times that of the isolated components; often it was 10 times that.

[3] Harrop, G. A., and Barron, E. S. G., *J. Exp. Med.*, 1928, **48**, 207.

[4] Barron, E. S. G., and Harrop, G. A., *J. Biol. Chem.*, 1928, **79**, 65.

[5] Barron, E. S., *J. Biol. Chem.*, 1929, **81**, 445.

[6] Warburg, O., Kubowitz, F., and Christian, W., *Biochem. Z.*, 1930, **227**, 245.

[7] Wendel, W. B., Proc. Soc. Exp. Biol. and Med., 1931, **28**, 401.

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DER ACETAT-VERONAL-PUFFER

VON L. MICHAELIS

(*Aus den Laboratorien des Rockefeller Institute for Medical Research*)

(Eingegangen am 11. März 1931)

In der vorangehenden Arbeit von *Kurt G. Stern* wurde von einem zusammengesetzten Puffer Anwendung gemacht, der ein weites p_H-Bereich zu umspannen gestattet, von p_H 2 bis 10, und leicht so eingerichtet werden kann, daß die „Ionenstärke" (ionic strength von *G. N. Lewis*) oder die „ionale Konzentration" (*N. Bjerrum*) konstant gehalten werden kann. Er hat außerdem den Vorteil, daß er nur einwertige Ionen enthält und daß er selbst im alkalischen Gebiet keine Fällung des Calciums erzeugt. Er ist eine Mischung von Acetat und dem kürzlich in die Puffertechnik eingeführten Veronal.[1]

Veronal ist bisher die einzige bekannte einwertige Säure[2] von einer Dissoziationskonstante in der Gegend von 10^{-8}, welche eine genügende Löslichkeit hat, um als Puffergrundsubstanz benutzt zu werden. Mit Mischungen von Veronal und seinem Natriumsalz umspannt man daher das p_H-Bereich, welches bisher allein von dem *Sörensen*schen Boratpuffer bedeckt wird. Dieser Boratpuffer hat schon manche verwirrende Resultate hervorgerufen, weil Borsäure die unangenehme Eigenschaft hat, mit allen möglichen organischen Substanzen, welche OH-Gruppen enthalten, komplexe Verbindungen zu geben. Das p_H-Bereich des Veronals überschneidet sich noch etwas mit dem Phosphatpuffer, so daß jetzt die Möglichkeit geschaffen ist, das physiologisch wichtige p_H 7,3 oder 7,4 außer mit Phosphatpuffer auch mit Veronal zu erzeugen. Günstig ist ferner, daß es

[1] *L. Michaelis*, Journ. of biol. Chem. **87**, 33, 1930.

[2] Wenn Veronal überhaupt imstande sein sollte, auch als zweiwertige Säure zu fungieren, so ist ihre zweite Dissoziationskonstante jedenfalls so klein, daß sie hier vernachlässigt werden kann.

nach einem früher beschriebenen Prinzip[1] leicht möglich ist, eine Veronalpufferreihe mit konstantem Salzgehalt (oder konstanter Ionenstärke im Sinne von *G. N. Lewis*) herzustellen, während dies für den Phosphatpuffer[2] viel schwerer und nicht ganz einwandfrei möglich ist.

Wenn man eine Stammlösung von Natriumacetat + Natriumveronal mit steigenden Mengen HCl versetzt, so kann man ein p_H-Bereich von 2 bis mindestens 9 umspannen, also das ganze Bereich, welches bei Fermentstudien in der Regel von Interesse ist, ohne daß man gezwungen ist, mit dem p_H gleichzeitig die Art der puffernden Grundsubstanzen sprunghaft zu ändern oder die Ionenstärke zu variieren, oder irgendein zweiwertiges Ion in dem Puffer zu verwenden.

Dieses Prinzip kann auf verschiedene Weise durchgeführt werden. Es soll hier eine Vorschrift gegeben werden, bei welcher die Ionenstärke durch passenden Zusatz von NaCl stets gleich einer für Blut isotonischen Salzlösung gehalten wird. Die Stammlösung ist eine Lösung, $\frac{1}{7}$ mol. sowohl in bezug auf Natriumacetat wie auf Veronal-Natrium: 9,714 g Natriumacetat, 3 H_2O und 14,714 g Veronal-Natrium werden in CO_2-freiem Wasser zu einem Volumen von 500 ccm gelöst. Von dieser Stammlösung werden je 5 ccm mit 2 ccm 8,5%iger NaCl-Lösung, mit *a* ccm n/10 HCl und (18—*a*) ccm H_2O versetzt. Die folgende Tabelle gibt die Beziehung von *a* und p_H auf Grund elektrometrischer Bestimmung mit der H_2-Elektrode bei 25,0°C.

a	p_H	*a*	p_H
(0	9,64)	7	6,12
0,25	9,16	8	5,32
0,5	8,90	9	4,93
0,75	8,68	10	4,66
1,0	8,55	11	4,33
2,0	8,18	12	4,13
3,0	7,90	13	3,88
4,0	7,66	14	3,62
5,0	7,42	15	3,20
5,5	7,25	16	2,62
6,0	6,99		
6,5	6,75		

[1] *L. Michaelis* u. *P. Rona*, diese Zeitschr. **27**, 38, 1910.
[2] *E. J. Cohn*, Journ. Amer. Chem. Soc. **49**, 173, 1927.

Durch Anwendung eines weiteren Überschusses an HCl kann man in das Wirkungsoptimum des Pepsins und darüber hinaus gelangen. Dann wird das p_H nur noch durch die überschüssige Salzsäure bestimmt.

Die Intervalle in der Tabelle sind entsprechend dem Wechsel in der Neigung der Kurve so gewählt, daß zwischen den angegebenen Intervallen eine lineare Interpolation erlaubt ist.

Natürlich muß, wie auch sonst stets, verlangt werden, daß das p_H in einer Versuchsmischung, zu welcher dieser Puffer zugesetzt wird, in jedem Falle besonders gemessen wird, da die Verschiebung des p_H durch die zu untersuchenden Materialien nicht allgemein vorausgesagt werden kann.

Nicht in allen Fällen wird die Aufrechterhaltung der Isotonie und Konstanthaltung der Ionenstärke in exakter Weise erforderlich sein. In diesem Falle kann man die NaCl-Lösung fortlassen oder durch H_2O ersetzen, ohne eine wesentliche Verschiebung des p_H befürchten zu müssen. In dieser Weise wurde in der vorangehenden Arbeit verfahren.

Zweifellos wird es Fälle geben, besonders bei Arbeiten mit lebendem Material, wo die narkotische Wirkung des Veronals die Anwendung des Puffers einschränkt, jedoch glaube man nicht, daß z. B. der Phosphatpuffer in dieser Beziehung ganz indifferent ist, wenn er nicht bis fast an die Grenze seiner Leistungsfähigkeit als Puffer verdünnt wird. Kritische Kontrollversuche müssen bei diesem wie bei jedem anderen Puffer vorausgeschickt werden.

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THE FORMATION OF SEMIQUINONES AS INTERMEDIARY REDUCTION PRODUCTS FROM PYOCYANINE AND SOME OTHER DYESTUFFS

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INTRODUCTION

In a recent study of the blue pigment of *Bacillus pyocyaneus* (1), it was observed that this dyestuff, in alkaline solution, can be reversibly reduced in the same way as any organic dye of the quinoid type, whereas in acid solution the reduction goes on in two successive steps, each characterized by an individual color and an individual potential. This behavior recalls the two step reduction observed by Willstätter and Piccard (2) for iminoquinone. But there is, according to what is known about the latter, a difference in the nature of the intermediary compound in the two cases. For iminoquinone the intermediary compound is considered by Willstätter as a molecular compound of the fully reduced and the fully oxidized states. This intermediary compound belongs to the quinhydrones, or more generally speaking, to what Willstätter and Piccard designated as meriquinones.[1] In contrast to this, in the case of pyocyanine the reduction goes on, according to the previous communication, in such a way that the intermediary compound is a true intermediary state of oxidation, with the same molecular size, without the formation of a molecular compound. In the case of pyocyanine, there is a simple reversible two step oxidation, each step involving the loss of 1 hydrogen atom. Such a case is, to my knowledge, encountered in the literature in one instance: Cannan (5) showed that hermidine, a vegetable pigment extracted from *Mercurialis perennis*, of un-

[1] However, *cf.* Hantzsch's (3) and Weitz and Fischer's (4) point of view as referred to in a later section of this paper.

known chemical constitution, is reduced in two successive steps, each involving the loss of 1 hydrogen atom and characterized by a particular color. Findings of this kind should not be considered as mere curiosities, but rather as something typical for certain dyestuffs. One cannot refrain from the speculation that this two step reduction must have a bearing both on the biological side of oxidation-reduction, and on the pure chemistry of dyestuffs at the same time. The first thing to do was to find some more dyestuffs of the same behavior. After several futile attempts two more dyestuffs were discovered which showed the same property. There can be little doubt that many others can be found[2] after the general principle of the matter is illuminated in a certain respect in this paper. One of these two is α-oxyphenazine, synthetized in 1928 by Wrede and Strack (6). It is worth mentioning that Wrede and Strack were unable to obtain the hydroxy compound by diazotizing Kehrmann's amino compound but had to prepare it in a quite different way. I am indebted to Professor Wrede of Greifswald for a specimen of his α-oxyphenazine. This dye is, according to Wrede and Strack, closely related to pyocyanine, which is nothing but a methylated α-oxyphenazine. The very surprising discovery of a phenazine derivative among the products of metabolism of a living organism seems to be confirmed beyond doubt by Wrede and Strack's synthesis of pyocyanine by methylizing α-oxyphenazine, in 1929. There is only one detail in which the formulation of pyocyanine of these authors seems to me inadequate, namely the bimolecular structure. The following experiments will show that there is no room for a bimolecular formula in pyocyanine in aqueous solution. The freezing point determinations by Wrede and Strack, which are in favor of the bimolecular formula, hold for organic solvents, but they prove nothing for aqueous solutions if the reasoning to be presented in this paper is acceptable. When $C_{13}H_{10}N_2O$ is taken for pyocyanine (the half of Wrede's formula) and $C_{12}H_8N_2O$ for α-oxyphenazine, according to Wrede's analysis in 1928, it becomes most likely that pyocyanine is nothing but monomethyloxyphenazine. The fact described by Wrede, that in strongly alkaline solution pyocyanine

[2] Several examples of this kind may be found in Hantzsch's paper (3). It is remarkable that these examples also belong to the derivatives of phenazine.

is partially converted to α-oxyphenazine, recalls the demethylation of methylene blue in alkaline solutions. No splitting of any polymerized form of pyocyanine need be assumed.

The other of the dyes is rosinduline and was studied in a previous communication (7) for the oxidation-reduction potential between pH 4 and 12. There was, then, no particular interest in extending this investigation to a more acid range. Now, however, this extension was performed, and in very acid solutions this dye showed the same behavior as pyocyanine.

It is a common feature of all these three dyes, pyocyanine, oxyphenazine, and rosinduline, that the splitting of the reduction process into two steps occurs only in acid solution. The two successive potential levels are more and more separated from each other as the pH becomes smaller. For pyocyanine, the separation begins to become manifest at pH about 5.5; for oxyphenazine at pH 3.8; for rosinduline not before pH 2.0. At pH values sufficiently smaller than the limiting values just given, the separation of the two potential levels is very distinct and no appreciable overlapping takes place. Each of the two steps of the titration curve has the appearance of a reversible oxidation system with the electron number 1, as it is in the case of ferricyanide-ferrocyanide, or ferripyrophosphate-ferropyrophosphate (8), and in contrast to the behavior of an ordinary organic dye in which what we called the electron number is always 2.

As to the common feature in the chemical constitution of these three dyes, at the present time the hint may suffice that all of them can be conceived of as derivatives of monoimidoquinone, $NH=C_6H_4=O$, in part of the orthoquinoid, in part of the paraquinoid type.

The difference between 1 molecule of each of the two successive steps will be proved to be only 1 electron (*i.e.* hydrogen atom) without any change in molecular size. The intermediary form will in this case be designated as a semiquinone. This is in contrast to that intermediary form which is designated as a quinhydrone, or more generally, as a meriquinone, and which is a molecular compound of the totally reduced form and the oxidized, holoquinoid form. We shall always in this paper use the words semiquinone and meriquinone with this distinction. The decision as to whether the intermediary form is a semiquinone or

a meriquinone can be attained by a mathematical analysis of the titration curve in the following way.

Theory of the Titration Curves for the Case of Semiquinone and of Meriquinone

The case in which the two step oxidation is due to the formation of a semiquinone can be expressed by Scheme A.

Scheme A

(1) $$XH_2 \rightarrow XH + H$$

(2) $$XH \rightarrow X + H$$

The case of the formation of a meriquinone can be expressed by Scheme B, where the meriquinone is supposed to have the simplest constitution, namely that of a quinhydrone (hydroquinone and holoquinone in the ratio 1:1).

Scheme B

(1) $$2\,XH_2 \rightarrow (XH_2, X) + 2H$$

(2) $$(XH_2, X) \rightarrow 2X + 2H$$

Depending on whether the reaction goes on according to Scheme A or B, the shape of the titration curve will be different. In all considerations we shall start with the completely reduced dye and titrate it with an oxidant. There will be two potential levels in either case. In general, the formula expressing the potential as a function of the amounts of the dye in its various possible forms of existence—the oxidized, intermediary, and reduced states—and of pH is extremely complicated. One may see from the paper on meriquinones by Clark and Cohen (9) how unhandy the formulæ become for the case of a meriquinone. They are not so very much simpler for the case of a semiquinone. It will not be necessary to develop these formulæ here, for the following reason. All difficulties entirely vanish if the two levels of the potential are distinctly separated; that is to say, if the oxidation of the intermediary form to the holoquinone does not begin, practically speaking, before the oxidation of the completely reduced form to the intermediary form has been finished. In all of our cases, this condition is fulfilled at a sufficiently low pH. We can restrict ourselves, therefore, to this case, and we may, furthermore, restrict ourselves to the consideration of the first half of the titration.

The second step, then, is a kind of repetition of the first. It will be a perfect repetition in the case of a semiquinone, except, of course, for the level of the potential. It will be what may be called an antisymmetric repetition in the case of a meriquinone, where each step of the titration curve is asymmetrically shaped around its particular mid-point. At any rate it is sufficient to analyze only one step of the oxidation in detail.

First Case. Formation of Semiquinone—Let the process go on according to Scheme A. Then the potential, during the first step of titration is simply this:

$$E = E_0 + \frac{RT}{F} \ln \frac{x}{a - x} = E_0 + 0.06 \log \frac{x}{a - x} \qquad (1)$$

where x is the amount of oxidant used for the titration, counted in equivalents (1 equivalent being that amount which accepts 1 gm. of hydrogen), and a the initial amount of the dye, in mols.

Second Case. Formation of a Meriquinone—Let the process go on according to Scheme B. Then the chemical reaction going on reversibly at the electrode, during the first step of oxidation, will be:

$$2\,XH_2 \rightleftharpoons (XH_2,\, X) + 2H^+ + 2\,\epsilon$$

and the potential will vary during the titration at constant pH according to the formula

$$E = E_0^* + \frac{RT}{2F} \ln \frac{[(XH_2,X)]}{[XH_2]^2}$$

If in the beginning XH_2 amounts to a mols, after addition of x equivalents of the oxidant it will be $a - X$; and $(XH_2,\, X)$ then will be $\frac{1}{2}\, x$. Hence

$$E = E_0^* + 0.03 \log \frac{\frac{1}{2}\,[x]}{[a - x]^2} = E_0^{**} + 0.03 \log \frac{[x]}{[a - x]^2}$$

$$E = E_0^{***} + 0.03 \log \frac{[x]}{[a - x]} - 0.03 \log\, [a - x] \qquad (2)$$

This formula shows that the potential depends not only upon the ratio of concentrations of the completely reduced form and the

meriquinone, but also upon absolute concentrations. The general shape of this curve will be somewhat complicated, but in general the slope of such a curve will lie somewhat between an ordinary titration curve for the electron number 1, and such for the electron number 2. In Fig. 6 one curve has been calculated for the electron number 1, the other for 2. The meriquinone curve would lie between those. Its curvature would fit, in its left-hand part more to the curve $n = 2$, in its right-hand part more to the curve $n = 1$. So the curve would be somewhat asymmetrically arranged about its middle ordinate. The experimental curve, however, fits entirely to the curve $n = 1$.

Furthermore the meriquinone curve would depend on absolute values not only on ratios of concentrations. The easiest way of formulating this is as follows: When in formula (2) the concentration $[a - x]$ is replaced by $\frac{(a - x)}{v}$, where the parenthesis means absolute amount in mols, and v volume, then

$$E = E_0^{****} + 0.03 \log \frac{(x)}{(a - x)} - 0.03 \log \frac{(a - x)}{v}$$

Keeping all variables constant except for v, one obtains:

$$E = \text{constant} + 0.03 \log v$$

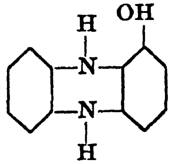
Let a given amount of the dye, amounting to a mols, be dissolved, for one experiment, in a definite volume of a buffer, and in another volume for another experiment. The dye is completely reduced and an oxidative titration performed for these two solutions. The two curves are plotted and compared, point after point. Then the potentials, for each particular value of x, in the two titration curves should differ by 30 millivolts when the volume of the one solution is 10 times that of the other. For practical purposes it was thought to be safer to compare one solution only with its 3-fold dilution, because the dye is so difficultly soluble that too high a dilution might have been disadvantageous for a rapid establishment of the potential. The difference of the potential should have amounted to $0.03 \times \log 3 = 0.0134$ volts. The experiment presented later on (Fig. 6), however, manifests no difference in the potentials at all in the two solutions.

According to Willstätter and Piccard (2), there is, furthermore, a possibility that a meriquinone is formed from its constituents in

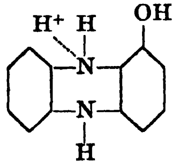
a ratio different from 1:1. On the supposition that this ratio was 1:2 or more, the displacement of the mid-point potential by variation of the volume would be still greater than in the case considered above. On the other hand, if this ratio were something like 2:3, the deviation of the whole curve from the one calculated for the ordinary case with the electron number of 1 would be still more conspicuous than in the simpler case. Thus, any kind of meriquinone formation can be excluded. This, of course, holds only for aqueous solution and need not be true for non-aqueous solutions or for the crystalline state.

Chemical Interpretation of the Intermediary Compound

Since the intermediary compound has been recognized as a semiquinone, the attempt has to be made to formulate such a compound. It must be a molecule of the character of a free radical, containing an odd number of electrons, like Gomberg's (10) triphenylmethyl, or Wieland's (11) di-aryl-nitrogen (see Lewis (12)). The following formulæ may serve as models for the case of oxyphenazine. Formula I is the completely reduced form: Formula I, a is the same as it exists in very acid solutions. It differs from Formula I in the same way as NH_4^+ from NH_3. Formula II is the semiquinone, which exists only in acid solution and contains what we may call bivalent nitrogen (comparable to Wieland's bivalent nitrogen). Formula III is the completely oxidized form of the holoquinoid type, in acid solution. In less acid or alkaline solution the hydrogen ion is released from the nitrogen, and Formula IV arises.



I



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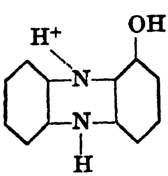

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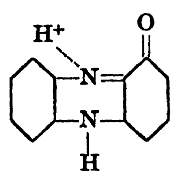
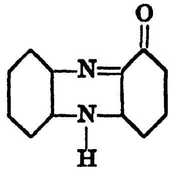
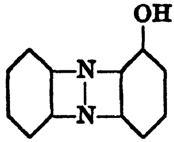


III



IV



V

In very alkaline solution a color change takes place indicating a further ionization of the oxidized form. This is made evident by applying Formula V which is tautomeric with Formula IV and contains a phenolic hydroxyl group. It is noteworthy that this tautomerism is not possible in the case of the methylated compound, pyocyanine. In accordance herewith, pyocyanine, in its oxidized form, shows no color change from a neutral to an extremely alkaline solution. (Something quite different and with no relation to this consideration is the fact that pyocyanine in very alkaline solution gradually undergoes an irreversible alteration attended by a change in color, as already described by Wrede and Strack.)

It is important to add that the radical-like compound, Formula II, is not a labile or metastabile molecule but is in true equilibrium with the other forms, and that it cannot release the attached hydrogen ion without losing its possibility of permanent existence.

It seems likely that on reinvestigation of those compounds now considered as meriquinones, by applying the methods proposed, some of these compounds will turn out to be semiquinones instead of meriquinones. At any event, this method may be used for further investigations on many of those intermediary oxidation-reduction products, as those compiled for instance in Henrich's book (13), and more recently discussed by Weitz (14), and linked with the names of Gomberg, von Baeyer, Pfeiffer, Kauffmann, Willstätter, Hantzsch, Kehrmann, Wieland, and many others. Especially, it should be pointed out that Weitz considers, quite generally, the quinhydrones not as molecular compounds but as radical-like single molecules, and that already Hantzsch (3) in 1916, strongly advocated a monomolecular formula for the quinhydrones. It may also be recalled that the potentiometric study by Conant, Small, and Taylor (15) on halochromic salts of triphenylmethyl belongs in this field.

It seems unlikely that the formation of a semiquinone as an intermediary product of reduction is restricted to the isolated cases presented in this paper. One may dare to express, as a working hypothesis for further investigations, the following idea. Quinoid substances may be able generally to form semiquinones. The conditions of their existence will be determined for aqueous solutions by a wedge-shaped field in a coordinate system, with pH and potentials as coordinates, in the same manner as the wedge designated as "green" in the figure in the paper on pyocyanine (1) or in Fig. 7 of this paper, or the field marked "violet" in Fig. 11 of this paper. The difficulty of generally proving this hypothesis may consist in the fact that the position of this wedge will often be in a part of the coordinate system which is not, or only difficultly, accessible for the experiment and where measurements of pH and oxidation-reduction potentials are uncertain. The wedge-shaped area may lie in a pH range corresponding to extremely concentrated strong acids, or in a potential range of overvoltage with respect to hydrogen or to oxygen. It seems likely that sometimes the area of existence of a semiquinone may lie outside the range of experimentally attainable conditions for aqueous solutions but inside the realizable conditions for non-aqueous solutions.

Description and Discussion of the Titration Curves

For the experiments with pyocyanine refer to the previous paper (1).

The methods for the two other dyes were the same as before. In all cases the dye was dissolved in the buffer solution. Both are only slightly soluble. With oxyphenazine the dye was dissolved in the gently heated buffer solution and filtered. With rosinduline the dye, in the form of the sodium salt, was dissolved in a drop of pure water, in which it is easily soluble unless pH is very low (< 3); then the acid (HCl or lactate buffer respectively) was added to the volume desired for the titration experiment. After the excess of dye had been precipitated in the constant temperature room at 30°, the solution was filtered and used for the titration. The solubility, at strongly acid reaction, is so low, that 20 cc. of the solution after reduction consumed altogether 0.70 cc. of 0.0005 M solution of quinone to complete reoxidation. Yet the potentials were fairly reproducible at different electrodes, at least in the well

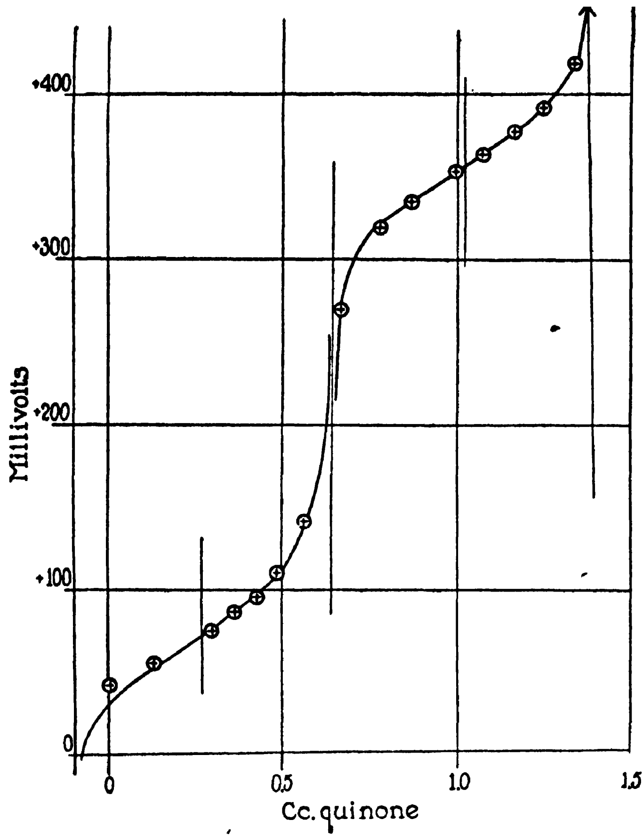


FIG. 1. α-Oxyphenazine, at pH 0.08 (approximately 1 N HCl), is completely reduced[3] and titrated at 30° with a quinone solution (likewise in 1 N HCl). The abscissa indicates cc. of the quinone solution added; the ordinate is the potential, in millivolts. All potentials in this paper are referred to the normal hydrogen electrode. The drawn out curve is, for each of the two steps in the potential level, the one calculated for a reversible oxidation with the electron number 1. Concerning the extrapolation of this curve to the negative side of the abscissa and the deviation of the first (or the two first) observed values from the theoretical curve see the text. The separation of the two halves is very distinct.

[3] During the state of complete reduction, in the atmosphere of pure hydrogen, the pH measurement was performed with a platinized electrode. All pH values in this paper are directly measured in this way; never was the pH value taken as it might have been approximately calculated from the composition of the buffer solution used. No correction, even in very acid solutions, was applied for the liquid junction potential between the buffer and the agar bridge saturated with KCl.

poised parts of the curves, though not quite with the same accuracy as was the case in the experiments with this dye at higher pH, as described in the previous paper, or with oxyphenazine in all pH ranges.

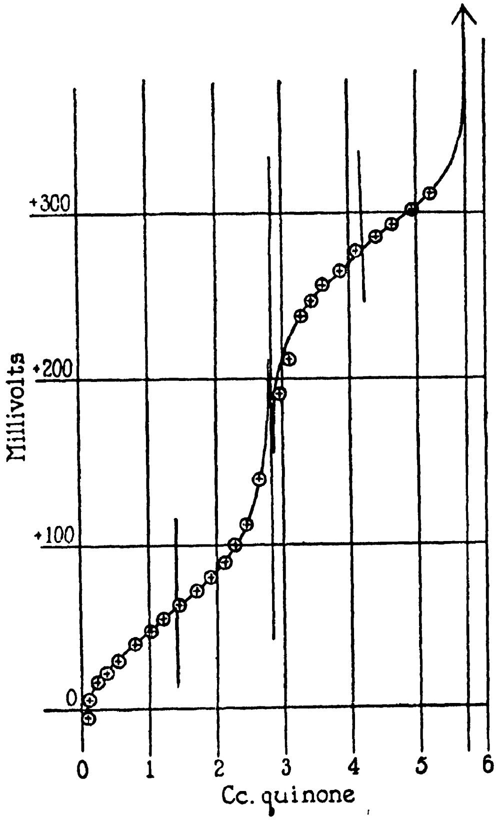


FIG. 2. α-Oxyphenazine dissolved in a solution of pH 1.00 (approximately 0.1 N HCl). Designations are the same as in Fig. 1. The separation of the two steps is very distinct here too, though somewhat smaller than in Fig. 1. The observed points fit into the calculated curve without any extrapolation being necessary.

The experiments with oxyphenazine are shown in Figs. 1 to 6 and summarized in Fig. 7. For those not accustomed to the methods applied it may be interesting to know that the total amount of dye consumed for all experiments, including the pre-

liminary trials and the losses by filtering off the undissolved residues of the difficultly soluble substance in each individual experiment, amounted to no more than 25 to 30 mg. Figs. 1 to 4 show the step formation at low pH ranges. The smaller the pH, the more distinctly are the steps separated. In Fig. 5, steps are no

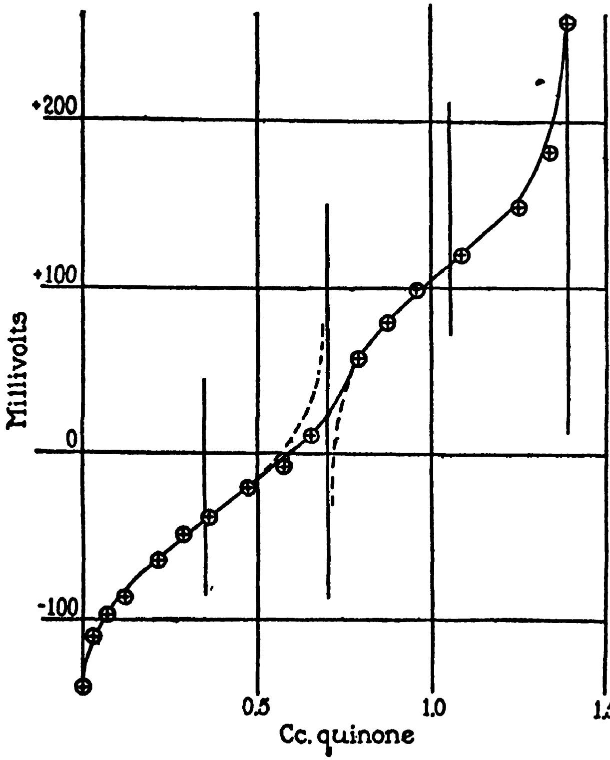


FIG. 3. α-Oxyphenazine at pH 1.858 (the substance is dissolved in about 1.5 cc. of 0.1 M HCl and the volume made up to 15 cc. with 0.1 M KCl solution). Designations are as in Fig. 1. The two steps of potential are here less distinctly separated so that in the middle part the observed curve can be imagined as an overlapping of the two theoretical curves.

longer separable, only the titration curve as a whole is somewhat too steep compared with a regular curve. The drawn out lines in Figs. 1 to 4 are those calculated for two not overlapping titration steps, each with an electron number of 1. The normal potential (*i.e.* the potential of the system in a half oxidized state), separately

shown for each of the two steps, is indicated by perpendicular lines halving each. In Fig. 6 it is shown that the titration curve is the same in every respect whether the original solution of the dye is titrated, or whether this solution is diluted three times. In the case of a meriquinone formation, the curves should differ in their level by 14 millivolts.

In the summarizing figure, Fig. 7, the normal potentials are plotted against pH. Between pH 2 and 9 the curve is rectilinear

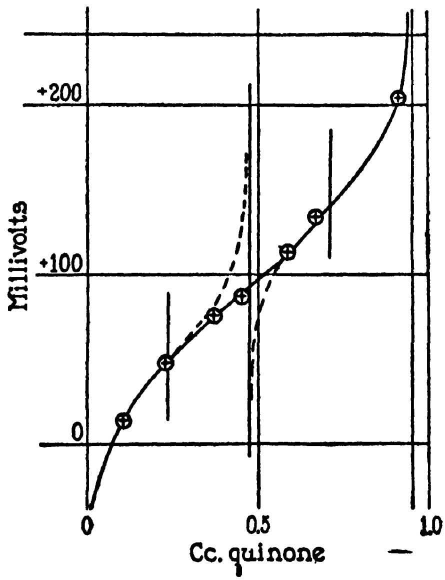


FIG. 4. α-Oxyphenazine at pH 2.640 (1 cc. of 1 M lactic acid and 20 cc. of 1 M sodium lactate). Designations are as in Fig. 1. Here the separation of the two steps is much less distinct; the overlapping is more pronounced. Yet the whole curve cannot be interpreted as an ordinary one step curve because the slope of the curve, taken as a whole, is much too steep.

with the usual slope of 0.06 volts per unit of pH. To the left-hand, the curve branches off, one branch being steeper, the other being flatter than before. The steeper branch has, in general, a slope of 0.09 volts per pH unit. But this branch is not simply a gradual shifting of the 0.06 slope. Rather there is undoubtedly a kind of soft bayonet or "S" form developed. The 0.06 slope leads, to the left-hand side, for a very short interval to a steeper

one, probably to 0.12 slope and then flattens a little to the 0.09 slope. One might be inclined to claim that this part of the curve should have been studied in even smaller pH intervals. There is, however, a difficulty in utilizing data closer together. In this part the overlapping of the two steps in each individual titration curve is so considerable, that the two normal potentials could be

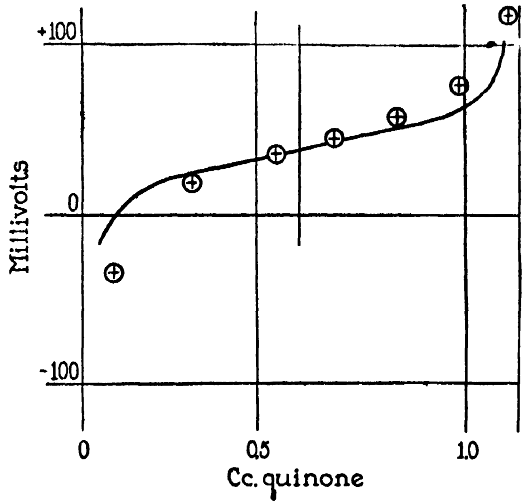


FIG. 5. α-Oxyphenazine at pH 3.472 (4 cc. of 1 M lactic acid + 2 cc. of 1 M sodium lactate, diluted to a volume of 20 cc.). Designations are as in Fig. 1. The drawn out curve, however, is here the one calculated for an oxidation in one step with the electron number 2. The overlapping of the two levels of oxidation is here so great that the only deviation of the observed points from the calculated curve is the fact, that the observed points form a curve just a little steeper than the drawn out curve. All titration curves at pH greater than in this experiment fit perfectly with the one calculated for a one step oxidation with the electron number 2. Since many of such examples have been shown in the previous papers, no further curves for oxyphenazine are described or plotted in detail.

only roughly estimated. This does not help, however, because only the most exact determinations of these normal potentials could lead to a definite decision as to whether or not the above interpretation of the slope is right. Anyhow, the 0.09 slope is precise, and so is the 0.06 slope; and, furthermore, there can be no doubt that the 0.06 slope does not shift smoothly to the 0.09 slope.

Now it is suggestive to correlate the inflections of the curve (Fig. 7) with the color changes of the dye. We begin with the ordinary, oxidized form of oxyphenazine, which corresponds to the field above the curve and, where the curve is branched, above

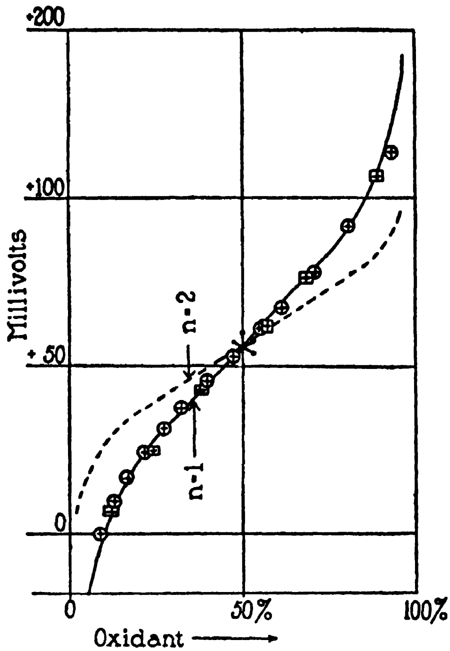


FIG. 6. This shows the independence of the potential of the concentration of the dye. α-Oxyphenazine at pH 1.382 (solution 0.05 M for HCl and 0.1 M for KCl). Only the first step of the oxidation is plotted. The abscissa indicates the amount of quinone, expressed in per cents of that amount which completes the first step of oxidation; the 'ordinates are potentials. The first experiment is represented by the circles. After finishing this experiment, the whole solution was diluted with the above acid mixture (containing no dye) to a three-fold volume, the dye re-reduced by H_2 + palladium, the hydrogen expelled, and the titration with quinone was repeated. It furnished the points marked with squares. The drawn out line is the one calculated for an oxidation with the electron number 1; the dash line the one calculated for the electron number 2.

the upper branch of the curve. The color is lemon-yellow from pH 2 to almost 8. To the left of 2 it turns over orange to pink, which is fully reached at pH of approximately -1 (HCl much

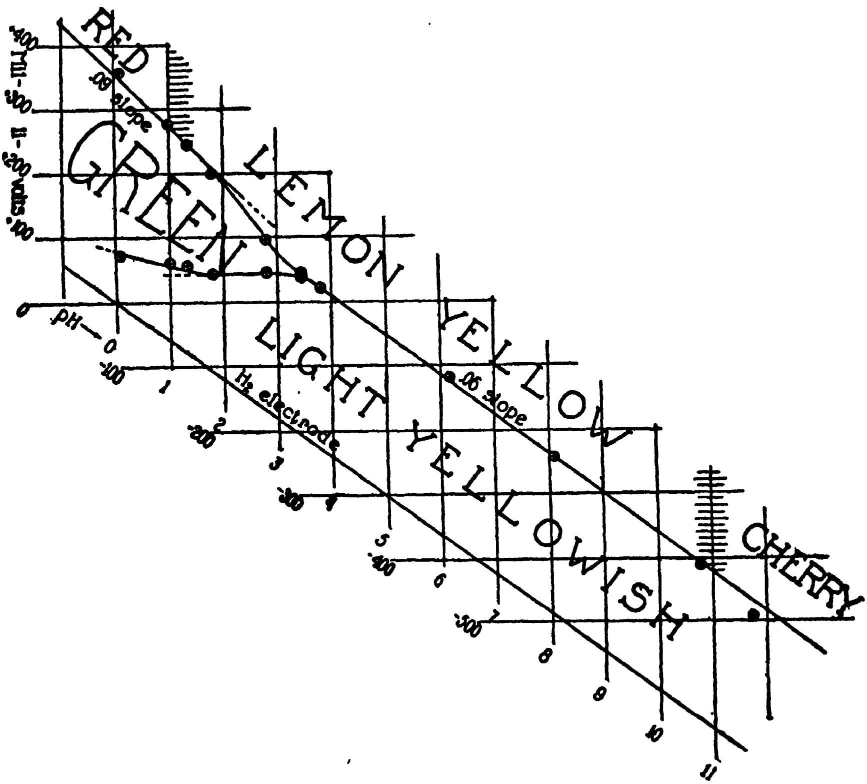


FIG. 7. The normal potential of α-oxyphenazine at varied pH. Abscissa, pH; ordinates, potential of the half reduced system. From pH about 4 to 12 the dye behaves as an ordinary organic dyestuff. The potentials plotted here are those of the half reduced state. At pH < 4, two levels of potential can be distinguished. The potentials plotted are here the potential of the half reduced state of each step of oxidation. The point at pH 3.4 corresponds to Fig. 5. The oblong mark of this point means the average value of two steps so close together that a separation into two distinct values cannot be executed with precision.

The color of the dye depends on pH and potential. In the oxidized form, the color varies from red to lemon-yellow to cherry-red according to pH. The transition zones of color are marked with cross-hatching. The completely reduced form is practically colorless, or slightly yellowish in higher concentrations. The wedge between the two branches of the curve is the area of the emerald green semiquinone. It should be noted that the lower branch of the wedge tends to intersect the hydrogen potential. The point of intersection, however, cannot be attained because of the enormous concentration of hydrogen ions at which it might be expected to occur.

stronger than 1 normal). To the right of pH 10.5 it turns over raspberry color to deep purple, which is fully reached in the pH range of a NaOH solution. There can be little doubt that the turning of the color around pH 0.5 signifies the ionization of one of the N groups, and the one around pH 11 indicates the ionization of a hydroxyl group which may be imagined to be present in the tautomeric Formula IV. The lemon-green color between pH 2 and 9 is the one of a broad isoelectric zone of the dye. It is noteworthy that during the inflections of the curve around pH 2 and 3 no change in color occurs. Neither can another step of dissociation be imagined beside the two mentioned. It can be inferred herefrom that the principles elaborated by Clark and Cohen (16) interpreting bendings in the curves in terms of dissociation constants cannot be simply applied to such a case. No explanation for these bends shall be offered now. This problem is worth a special study and, being of no importance for the subject of this paper, will not be discussed here.

The intermediary step of reduction as framed by the wedge formed by the branches of the curve is always deep emerald green. The unsaturated condition of the nitrogen has a bathochromic effect. The completely reduced form, covering the field below the curve, is always colorless (or very slightly yellowish when in higher concentrations).

In the case of pyocyanine the transition zone of the oxidized form of the dye from blue to red (pH 4.9) did not agree with the beginning of the branching (pH 5.8) nor did it manifest itself by a bending of the curve, as one sees from Fig. 7 in the previous paper (1).

A special discussion, furthermore, is required for the experiment in Fig. 1, at pH 0.08. As one sees from the summarizing Fig. 7 the normal potential of the first step of oxidation is here rather close to the potential of hydrogen of 1 atmosphere pressure at the same pH. Under this condition, the reduction of the dye by palladium and hydrogen of 1 atmosphere pressure cannot be strictly complete. According to the value of this potential as plotted in Fig. 7, the reduction can go on only to 88 per cent. In fact, the reduction by hydrogen gas plus palladium did not proceed to completeness and a residue of the green color of the intermediary state remained permanently. When the hydrogen was

bubbled out and replaced by nitrogen, the green became even more obvious. Here the change of potential, as produced from the mere diminution of the hydrogen pressure, overlaps with the titration curve of the dye. Therefore, the very first steps of the titration curve had to be performed somewhat hastily until steady conditions were reached. In order to evaluate the normal potential as precisely as possible, the actual titration curve of the first step had to be replaced by an idealized curve with no overlapping with the hydrogen potential. This idealization could be per-

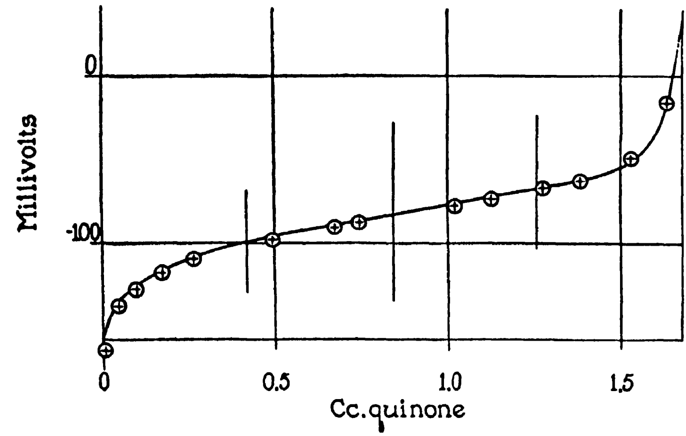


FIG. 8. Rosinduline, dissolved in lactate buffer, pH 3.821, completely reduced, and titrated with quinone. Abscissa, cc. of quinone solution; ordinate, potential, referred to the normal hydrogen electrode. The drawn out curve is the one calculated for an electron number 2, such as in any ordinary dye of the quinoid type.

formed in the following way. The second step of the titration is without such objections, and should use the same amount of oxidant as the first. So we can infer from this second step how many cc. of the oxidant should have been necessary for the first step if the overlapping had not occurred. By this extrapolation the idealized drawn out curve as shown in Fig. 1 was obtained, and the normal potential computed graphically from this theoretical curve. It may be added that this extrapolation is very small, and that even an appreciable error in the graphic construction of this curve would involve an error only of a few millivolts for the value of the normal potential. One has, besides, to take into

consideration that in this range of pH the pH determination itself might not be absolutely strict on account of the liquid junction potential between so acid a solution and the KCl bridge.

No titration curve is printed for any pH > 4. They fitted precisely the theoretical curve for a regular dye such as shown in several preceding publications.

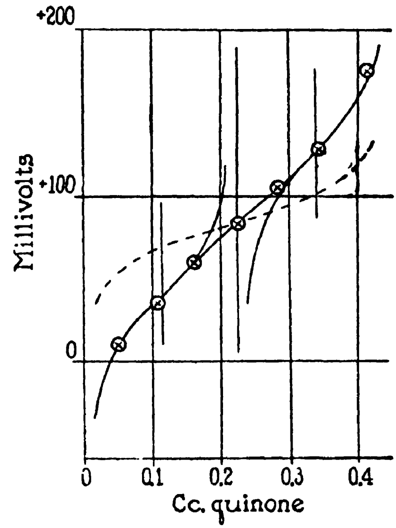


FIG. 9. Rosinduline dissolved in approximately 0.1 N HCl, pH 1.081. Abscissa and ordinates are as in Fig. 8. The dash line curve is the one calculated for the electron number 2; it does not fit at all. The drawn out curve is composed of two half curves, each calculated for the electron number 1, the overlapping part is graphically smoothed so as to form one single curve. In this case, the step formation is graphically not so clear. The whole curve might have been interpreted as a single step curve with the electron number 1. This interpretation, however, would not account for the fact that the color goes from colorless[4] through violet to orange, the violet being most intense and most pure at the mid-point of the titration.

For rosinduline, Figs. 8 to 10 show some individual titration curves. For the curves at higher pH, Fig. 8 is an example. The drawn out curve is calculated for the electron number 2. Figs. 9 to 11 show the formation of the two steps in acid solutions. Fig.

[4] The completely reduced form is sometimes designated as colorless, sometimes (as in Fig. 11) as light yellow. It is, in fact, so light in color that in so low a concentration as used in the experiment, Fig. 9, it appears colorless.

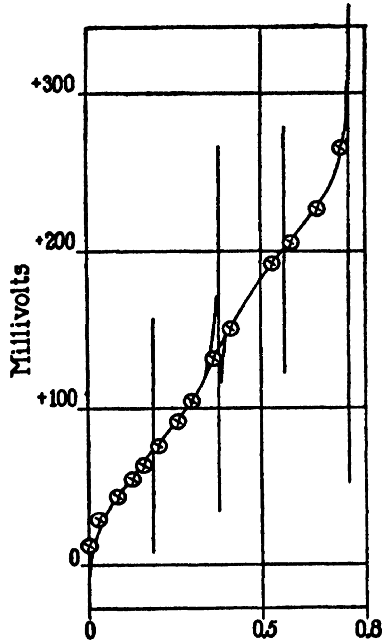


FIG. 10. Rosinduline, dissolved in approximately 1.0 N HCl, pH 0.080. Designations are as in Fig. 9. Here the step formation is already distinct. The two halves of the curve are calculated each for the electron number 1. The color, during the titration, turns from colorless over violet to orange.

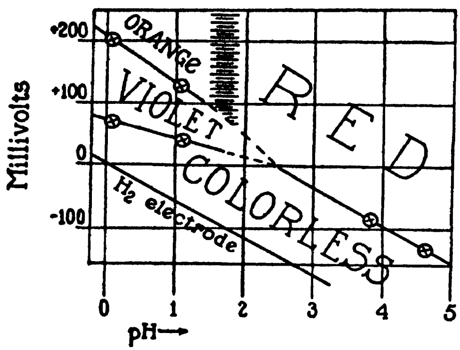


FIG. 11. Normal potential of rosinduline at varied pH. Abscissa, pH. The abscissa is drawn only from pH 0 to 5. An extension of this plot is the diagram Fig. 1 in the previous paper (7). Ordinate, potential of the half reduced dye, referred to the normal hydrogen electrode. The oxidized form (above the curve) is red, respectively orange, according to pH; the transition being marked by dashes. The intermediary form is violet, the reduced form very light yellow.[4]

11 summarizes the results. It may be mentioned that the point for pH 3.82 (Fig. 8) (lactate buffer) has been added after the previous communication (7) and fits in the summarizing diagram (Fig. 11) precisely into the 0.06 slope which this dye shows from very alkaline up to very acid solutions until finally the branching of the slope starts at pH about 2.5. Fig. 11 summarizes the results only for acid solutions, because the slope for higher pH has previously been published in Fig. 1 of a previous paper (7).

SUMMARY

For three dyestuffs, pyocyanine, α-oxyphenazine, and rosinduline, it is shown that between the oxidized and the reduced state there exists an intermediary state, provided the solution is very acid. This intermediary state is recognizable by its particular color. It has been proved by an analytical treatment of the titration curves that this intermediary state is not a meriquinone (a molecular compound of the reduced and the oxidized forms) but a non-polymerized, half reduced state with the character of a free radical. This intermediary state may be designated as a semiquinone, in distinction to a meriquinone. The conditions for its coexistence in a true equilibrium with the other forms of a quinone-hydroquinone system are discussed. This existence depends both on pH and on the oxidation-reduction potential of the solution. These two variables being used as coordinates, the existence of the semiquinone is determined by a wedge-shaped area.

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ÜBER DIE AUTOLYTISCHE WIRKSAMKEIT DER TIERISCHEN GEWEBSPROTEINASEN UND IHRE BEEINFLUSSUNG DURCH SCHWERMETALLE

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INHALTSÜBERSICHT

I. EINLEITUNG

Das Phänomen der Aktivierung der proteolytischen Fermente der sogenannten dritten Gruppe, zu denen u. a. die pflanzlichen Proteinasen *Papain* und *Bromelin*, ferner die *Hefeproteinase* und das in allen tierischen Zellen aufgefundene *Kathepsin* zählen, mittels Blausäure, Schwefelwasserstoff und weiterer Substanzen, denen die Fähigkeit gemeinsam ist, mit Schwermetallen Komplexe zu bilden, ist verschiedenartig interpretiert worden.

Die genannten Aktivatoren wurden von der *Willstätter*schen Schule als cofermentartige Körper aufgefaßt, die sich mit der Proteinase unter Erweiterung der Spezifität und Verstärkung der Aktivität analog dem System Enterokinase-Trypsin

verbinden. Hierfür scheint vor allem die zur Erreichung einer maximalen Aktivierung erforderliche Inkubationszeit zu sprechen. Auf der anderen Seite glaubt *Krebs*, durch seine Untersuchungen am *Papain* den Mechanismus der Aktivierung als *scheinbaren* Effekt entlarvt zu haben, hervorgerufen durch die Anwesenheit vergiftender Schwermetallspuren in den Enzym- oder Substratlösungen, die durch die zugefügten Komplexbildner unschädlich gemacht würden. *Krebs* findet, daß Papain durch die Schwermetalle der ersten und zweiten Vertikalreihe des periodischen Systems vergiftet wird, wobei die hemmende Wirkung innerhalb einer Gruppe mit zunehmendem Atomgewicht ansteigt. Die übrigen untersuchten Schwermetalle, besonders Eisen, waren nach *Krebs* ohne Einfluß. Es muß allerdings darauf aufmerksam gemacht werden, daß diese Versuche im Hinblick auf die benutzte *manometrische* Methodik weit abseits vom p_H-Optimum des Papains ausgeführt wurden. Nachdem dieser Autor nun an einem Beispiel gezeigt hat, daß Kaninchenmilzkathepsin ebenfalls durch Kupfer vergiftet wird (Bestimmung der aus Gelatine freigelegten NH_2-Gruppen nach *van Slyke*), kommt er zu dem Analogieschluß, daß sich dieses Enzym gegen Schwermetalle gleichartig wie Papain verhält. Wie im folgenden gezeigt wird, ist es zwar richtig, daß Kaninchenkathepsin durch Kupfer vergiftet wird, doch können die Kathepsine anderer Tierarten durch Kupfer, Zink, Mangan und Eisen in einer Reihe von Fällen nicht nur nicht geschädigt, sondern im Gegenteil erheblich aktiviert werden.

In der älteren Literatur finden sich zahlreiche Beobachtungen, wonach die Wirksamkeit der autolytischen Fermente, die nunmehr als die normale proteolytische Ausrüstung der Tierzelle (Kathepsin) erkannt worden sind, durch eine Reihe von Schwermetallen in verschiedenem Sinne beeinflußt wird. So ist z. B. von *Truffi* der fördernde Einfluß von Eisen- und Quecksilbersalzen in geringer Konzentration, von *Preti* Aktivierung durch Blei-, von *Izar* Förderung durch Silbersalze beschrieben worden. *Bradley* berichtet über Aktivierung der Autolyse durch Mangan, *Ascoli* durch eine Reihe kolloider Metalle, besonders durch Eisen-, Silber- und Kupfersole. Andere Autoren wieder fanden verschiedenartige Beeinflussung normaler und pathologischer Gewebe durch gleiche Zusätze (z. B. *Fasiani* durch Selen und Tellur).

Besonders erwähnt seien die mit exakter Methodik durchgeführten Versuche von *Rona* und *Mislowitzer* über den Einfluß von Erdalkalien und Alkalien, sowie einer Reihe von Anionen auf die Zellproteinase, aus denen hervorgeht, daß sich die Effekte der genannten Ionen oft abseits vom optimalen p_H umkehren.

Da gegen die älteren Arbeiten über Einflüsse von Schwermetallen auf die Autolyse der Einwand erhoben wurde, daß die beobachteten Effekte zum Teil auf p_H-Verschiebungen infolge Arbeitens in ungepufferten Systemen, zum Teil auf Verunreinigungen zurückgeführt werden könnten, wurde hier versucht, die Einflüsse verschiedener Zusatzstoffe, besonders einiger Schwermetallsalze, ferner von Selen und einigen Metallkomplexen auf die Wirksamkeit des Kathepsins unter gut reproduzierbaren Bedingungen zu studieren.

Es wurde demgemäß der Abbau von Gewebseiweiß, das sich in Organ auszügen

verschiedener Herkunft gelöst befand, durch die in den gleichen Extrakten enthaltene Zellproteinase unter dem Einfluß der genannten Stoffe untersucht. Es gelang, unter Zuhilfenahme der *nephelometrischen* Analyse, diese „*autolytische Reaktion*" innerhalb kurzer Versuchszeiten (5 bis 180 Minuten) in homogenen und gut gepufferten Systemen über einen weiten Spaltungsbereich hinweg messend zu verfolgen. Im folgenden seien die bei vergleichender Untersuchung einer Reihe von Organen verschiedener Tierarten erhobenen Befunde beschrieben. Einer späteren Veröffentlichung soll ein weiteres Eingehen auf den Mechanismus und die Kinetik der Reaktionen vorbehalten sein. Ebenso soll das entsprechende Verhalten der *Hefeproteinase*, die ebenfalls unter die katheptischen Enzyme fällt, späterhin beschrieben werden.

II. ALLGEMEINER TEIL

In ihrer Studie der proteolytischen Enzyme der Milz haben *Waldschmidt-Leitz* und *Deutsch* essigsaures Glycerin für die Extraktion der Proteasen angewendet. Nach ihren Feststellungen enthält ein solcher Auszug nur eine einzige echte Proteinase, von *Willstätter Kathepsin* benannt, die im schwach sauren Gebiet optimal auf zugesetzte Proteine wirkt, während die in relativ viel größerer Menge vorhandene Ereptase Peptide optimal bei p_H 8 spaltet. *Kleinmann* und *Stern* bestätigten diesen Befund in vollem Umfange mit Hilfe der nephelometrischen Analyse und konnten die Proteinase mittels Adsorption an Kaolin und folgender Elution mit alkalischen Puffern in gereinigtem Zustande herstellen und ihr Verhalten gegen zugesetzte Substrate, nämlich Gelatine, Serumalbumin und Casein studieren. Dabei wurde ein Wirkungsoptimum des Kathepsins auf diese Proteine zwischen p_H 3,5 und 4,5, je nach dem benutzten Substrat und dem Reinheitszustande des Enzyms, festgestellt. Kürzlich wurde das proteolytische System der Tierzelle durch Auffindung einer Carboxy- und einer Amino-polypeptidase durch *Waldschmidt-Leitz* und Mitarbeiter vervollständigt.

Die nach *Waldschmidt-Leitz* hergestellten normalen Glycerinauszüge aus tierischen Organen enthalten natürlich neben den Proteasen noch eine Reihe Begleitstoffe, darunter durchschnittlich 0,8% an Organeiweiß. Dieses liefert mit Sulfosalicylsäure und Salzsäure eine ausreichend stabile und gleichförmige Trübung, die zur Ermittlung der Konzentration nephelometrisch ausgewertet werden kann. Während das in den Extrakten befindliche Eiweiß während des Lagerns der Auszüge im Kühlschrank durch die hohe Glycerinkonzentration und die tiefe Temperatur vor dem Angriff der proteolytischen Enzyme

monatelang geschützt bleibt, setzt nach entsprechender Verdünnung, Einstellung der optimalen Wasserstoffionenkonzentration mittels Regulatoren und Erwärmung auf über 30° ein rapider Abbau des Substrateiweißes ein, der mittels der obengenannten Trübungsreaktion durch Probeentnahme in geeigneten Intervallen bequem messend verfolgt werden kann. Die Geschwindigkeit des Prozesses sinkt, nachdem innerhalb der ersten 2 Stunden nach Versuchsbeginn meist über 60% des vorhandenen Substrats in tiefere Produkte aufgespalten worden sind, rasch ab, und selbst nach 48 Stunden sind selten mehr als 75% der Proteinmenge verschwunden.

Die vorhandenen Proteinmengen im Auszug sind so klein, daß mit einer weniger empfindlichen Methodik, wie z. B. der Ermittlung des Zuwachses an Amino-N nach *van Slyke*, merkliche Ausschläge erst nach 24 Stunden Spaltzeit unter Verwendung der vielfachen Extraktmenge erhalten werden. (Siehe dazu *Kleinmann* und *Stern*, I. Mitteilung über Gewebsproteasen.)

A. Anzahl und optimale p_H-Aktivität der Gewebsproteinasen

Es wurde eine Reihe gleichartiger Auszüge verschiedener Organe verschiedener Spezies bereitet und mit Hilfe der S. 317 beschriebenen Technik eine p_H-Aktivitätskurve der autolytischen Reaktion aufgenommen. Die gefundenen Optima sind in der Tabelle I zusammengestellt. Es geht daraus hervor, daß, bis auf den Fall der Kaninchenniere und der Fischleber und -milz, in den untersuchten Gewebsauszügen nur eine einzige echte Proteinase zugegen war.

In der *Kaninchenniere* wurden zwei im sauren Gebiet liegende Wirkungsoptima gefunden, eines bei p_H 3 und das zweite bei p_H 4,6. Da sich in der Literatur Angaben fanden (s. bei *Oppenheimer*), daß kleine Magenpepsinmengen mit dem Harn ausgeschieden werden, wurde Kaninchenharn in seinem Verhalten gegen zugesetztes Serumalbumin geprüft und in der Tat eine optimal bei p_H 3 wirksame Proteinase festgestellt. Da in der Kaninchenleber nur ein einziges Optimum, nämlich das Kathepsinoptimum bei p_H 4, beobachtet wurde, dürfte erwiesen sein, daß es sich bei dem ersten Wirkungsoptimum des Nierenextraktes nicht um ein echtes Zellenzym, sondern um die im Urin ausgeschiedene Protease handelt, die bei der Extraktion des Organs in die Extrakte überging.

Auch im Falle der *Fischorgane* (Karpfenleber und -milz) ist an dem Vorhandensein nur *einer* echten Gewebsproteinase nicht zu zweifeln: Die histologische Untersuchung der zur Extraktion verwendeten Organe ergab, daß sowohl im Milz- wie im Lebergewebe zahlreiche

TABELLE I

Übersicht über die optimale Wasserstoffionenkonzentration für die Autolyse in verschiedenen Organauszügen

Spezies	Organ	Optimales p_H
Mensch	Leber	3,6
	Milz	3,2
Pferd	Niere	3
	Milz	4
Kalb	Leber	4
	Niere	4
	Milz	4
	Muskel	4
Kaninchen	Leber	4
	Niere	3 und 4,6
	(Urin	3)
Ratte	Leber	3,5
	Niere	4
	Cori-Tumor	4
Huhn	Leber	4
Frosch	Leber	4
Karpfen	Leber	4 und 9–10
	Milz	4,6 und etwa 9

verstreute Inseln von Pankreasgewebe zugegen waren (Lienopankreas und Hepatopankreas), die offenbar daß in den Auszügen gefundene tryptische Enzym sezernieren. Bemerkenswert ist, daβ das Wirkungsoptimum dieser Tryptase alkalischer ist als das der bisher beschriebenen Tryptasen.

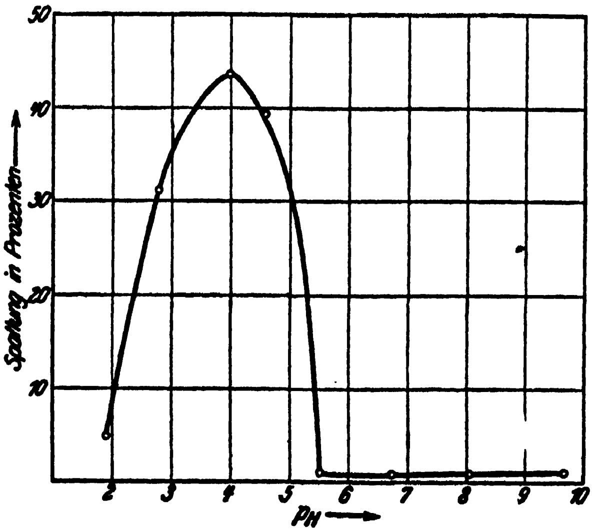


ABB. 1. Aktivitäts-p_H-Kurve der Kaninchenleberextraktautolyse als typisches Beispiel für die Aktivität der einzigen echten Zellproteinase (Kathepsin).

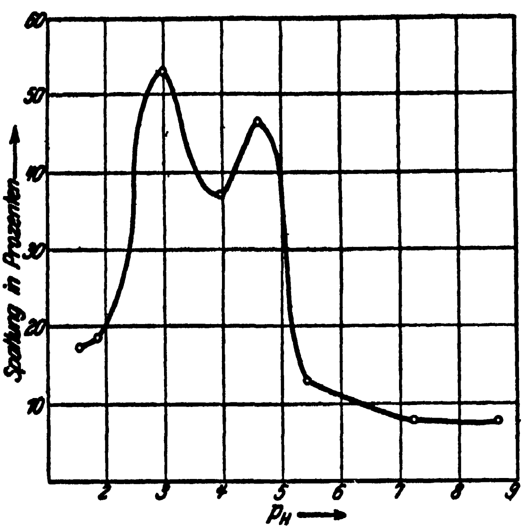


ABB. 2. Aktivitäts-p_H-Kurve der Kaninchennierenextraktautolyse: Das erste Maximum (bei p_H 3) kommt dem Harnpepsin, das zweite Maximum (bei p_H 4,6) kommt dem Nierenkathepsin zu.

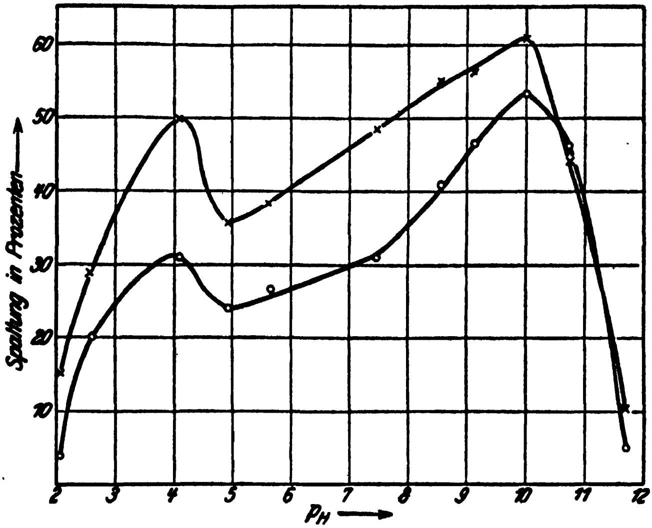


ABB. 3. Aktivitäts-p_H-Kurve der Karpfenleberextraktautolyse: Das im sauren Gebiet (bei p_H 4) gelegene Wirkungsoptimum entspricht dem Leberkathepsin, das alkalische Optimum (bei p_H 10) ist auf sezernierte Pankreastryptase zurückzuführen. Die untere Kurve stellt den Substratumsatz nach 15 Min., die obere den Umsatz nach 45 Min. Versuchsdauer dar.

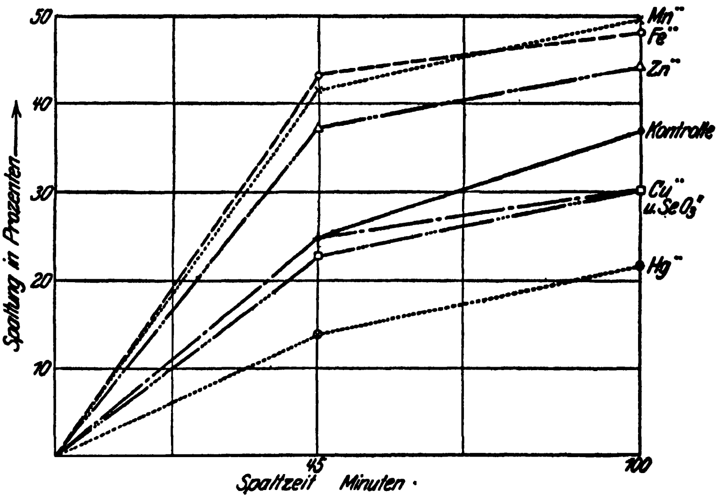


ABB. 4. Die verschiedenen Kurven geben den Ablauf der Menschenleberextraktautolyse unter dem Einfluß von Cu¨, Zn¨, Hg¨, Mn¨, Fe¨ und SeO_3''-Ionen sowie des Kontrollversuchs ohne Zusatz wieder.

B. Einfluß verschiedener Schwermetall- und Selenitionen auf die Extraktautolyse

Um die Beeinflussung der autolytischen Reaktion in den Organauszügen zu studieren, wurden die Extrakt-Puffermischungen bei der optimalen Wasserstoffionenkonzentration für die betreffende Proteinase mit (für die verschiedenen Auszüge gleichen) Metallsalz- bzw. Selenitlösungen versetzt, deren Quantität in einem Vorversuch (mit Kalbsmilzextrakt) als wirksam befunden worden war. (Technik siehe S. 318.)

Die gewählten Konzentrationen der Zusatzstoffe lagen durchschnittlich etwa um eine Zehnerpotenz über den von *Krebs* in seinen Versuchen mit Papain angewandten.

Die hierbei erhaltenen Resultate sind in der Tabelle II zusammengestellt. Aus der Zusammenstellung ergibt sich, daß sich die Gewebsproteinasen durchweg verschieden gegen die gleichen Konzentrationen der Zusatzstoffe verhalten, wobei jedoch die Differenzen zwischen den Organen der gleichen Spezies meist viel geringer sind als diejenigen zwischen verschiedenen Spezies. Im allgemeinen wurde gefunden, daß Ferro- und Manganoionen aktivierend, Quecksilber-, Kupfer- und Selenitionen meist hemmend wirkten, während Zinkionen in einigen Fällen aktivierten, in anderen dagegen hemmten. In vielen Fällen waren auch die zugesetzten Stoffe ohne jede Wirkung auf die untersuchte Reaktion.

Interessant erscheint das gleichartige Verhalten der Extrakte aus Cori-Rattentumoren und aus Rattenlebern.

Lebertrypsin und Leberkathepsin der Fische, die ja auf das gleiche Substrat einwirkten, unterscheiden sich besonders in ihrem Verhalten gegen Quecksilber. Die Tryptase ist das einzige der untersuchten Enzyme, welches durch Quecksilber etwas aktiviert wurde.

In dem Verhalten der einmal aus vorher *entbluteter* und das andere Mal aus *bluthaltiger* Kaninchenleber bereiteten Auszüge besteht kein erheblicher Unterschied.

Kaninchenleber- und -nierenkathepsin auf der einen Seite und Kaninchenmagen-, Nieren- und Harnpepsinase auf der anderen Seite können dadurch voneinander unterschieden werden, daß die erstere Gruppe durch die gleiche Konzentration an Zinksalz deutlich gehemmt wird,

TABELLE II

Effekt von Kupfer-, Zink-, Mercuri-, Mangano-, Ferro- und Selenitionen auf die autolytische Aktivität von Gewebsproteinasen, ausgedrückt in Prozenten der Aktivität der unbeeinflußten Enzyme

Spezies	Organ	$Cu^{\cdot\cdot}$ $7\cdot 10^{-2}$ mg/ccm	$Zn^{\cdot\cdot}$ $8\cdot 10^{-2}$ mg/ccm	$Hg^{\cdot\cdot}$ $14\cdot 10^{-2}$ mg/ccm	$Mn^{\cdot\cdot}$ $10\cdot 10^{-2}$ mg/ccm	$Fe^{\cdot\cdot}$ $7\cdot 10^{-2}$ mg/ccm	SeO_3'' $25\cdot 10^{-2}$ mg/ccm
		%	%	%	%	%	%
Mensch	Leber	86	135	57	150	155	91
	Milz	93	125	60	130	120	86
Pferd	Niere	96	180	43	220	185	56
	Milz	81	120	50	120	122	81
Kalb	Milz	135	190	61	190	190	100
	Niere	100	135	90	140	135	72
	Leber	100	140	70	165	150	69
Kaninchen	Niere:						
	1. Magen = Harnpepsin im Nierenextrakt	48	100	70	110	100	57
	2. Kathepsin	18	54	36	68	70	32
	Leber:						
	1. entblutet	43	70	36	100	100	34
	2. mit Blut	50	57	57	100	100	30
	Harnpepsin*	55	100	33	100		100
	Magenpepsin	72	100	65	130	100	69
Ratte	Leber	100	140	78	150	145	66
	Cori-Tumor	89	135	82	152	140	89
Huhn	Leber	36	100	26	127	118	28
Frosch	Leber	21	100	21	115	106	20
Karpfen	Milzkathepsin	24	68	26	81	78	33
	Leberkathepsin	72	86	91	105	100	70
	Pankreastryptase im Leberextrakt	100	75	113	100	100	100

* Als Substrat wurde hierbei Serumalbumin vom Rinde verwendet.

während die letztere Gruppe, wie aus Abb. 6 hervorgeht, durch die gleiche Konzentration eher etwas aktiviert wird.

Es sei noch erwähnt, daß sich in einem Versuch mit Kalbsmilzextrakt Gold ($6 \cdot 10^{-2}$ mg/ccm) als hemmend, Arsenit ($9 \cdot 10^{-2}$ mg/ccm) und Tellurat als wirkungslos erwiesen.

Da der aktivierende Einfluß von *Eisen*, wie er z. B. bei Organ-extrakten von Mensch, Pferd, Kalb und Ratte gefunden wurde, physiologisch von besonderem Interesse ist, wird augenblicklich auch der Effekt von

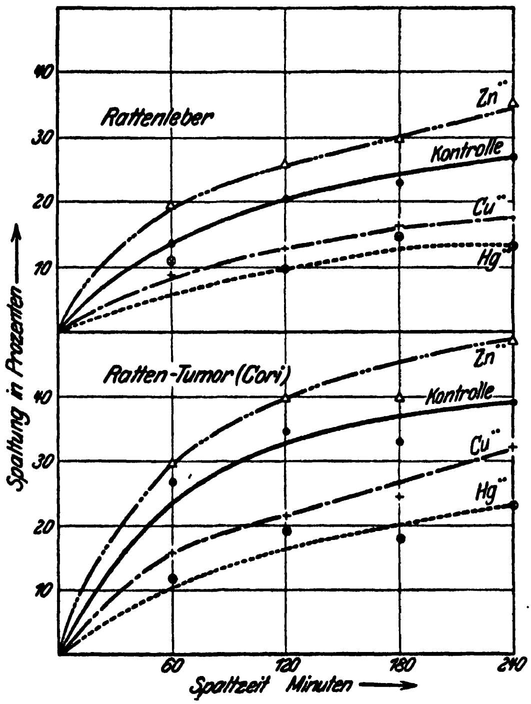
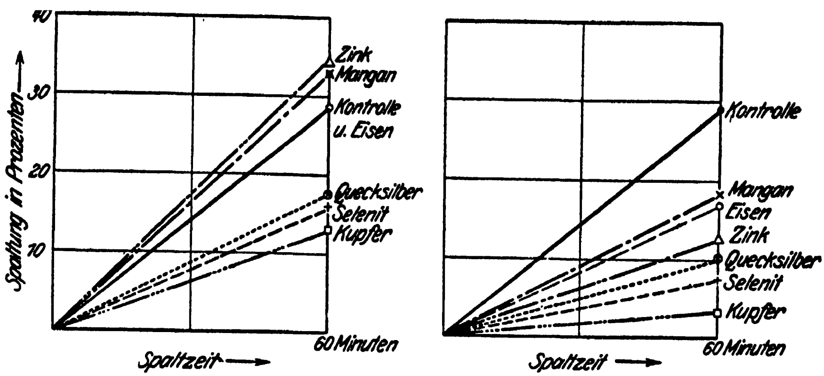


ABB. 5. Die beiden Diagramme veranschaulichen das gleichsinnige Verhalten der Rattenleber und Cori-Rattentumor-Extraktautolyse gegen Zink-, Kupfer- und Quecksilberzusatz.

komplex gebundenem Eisen studiert. Es sei hier aus einer folgenden Mitteilung bereits vorweggenommen, daß der Eisen-Blausäurekomplex auf die Kalbsmilzextraktautolyse ohne Einfluß war, während sich sowohl Eisen- wie Zink in dem Komplex mit *Dipyridyl* stärker aktivierend erwiesen als die gleichen Metallmengen in Ionenform.

Der aktivierende Effekt von Zinksalz auf die Kalbsmilzextraktautolyse unter Variierung der Wasserstoffionenkonzentration ist in der



Harnpepsinase im Kaninchennierenextrakt

Kathepsin der Kaninchenniere

Abb. 6. Die Abbildung demonstriert das verschiedenartige Verhalten der auf das gleiche Substrat (Niereneiweiß) einwirkenden Pepsinase und des Kathepsins (enthalten im Kaninchennierenextrakt) gegen gleiche Konzentrationen verschiedener Zusätze. Das Kathepsin wird durch alle zugesetzten Salze mehr oder weniger gehemmt, während die Pepsinase durch Zink und Mangan eher etwas aktiviert wird. (Aktivitäs-p_H-Kurve der beiden Enzyme siehe Abb. 2.)

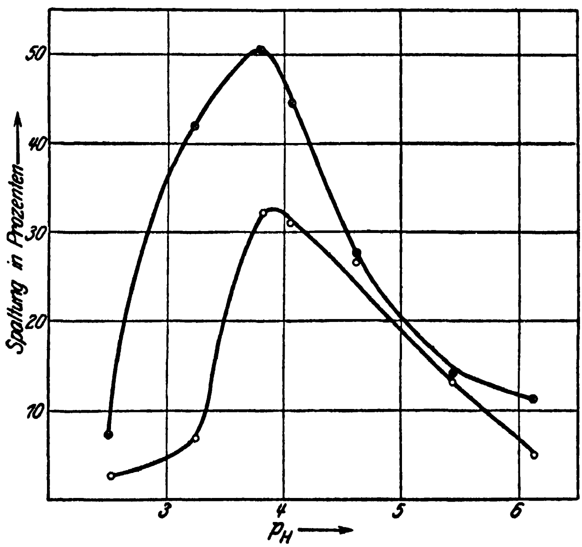


Abb. 7. Effekt des Zinks auf den Ablauf der Kalbsmilzextraktautolyse bei variiertem p_H. Die untere Kurve entspricht dem Kontrollversuch ohne Zinkzusatz.

nachstehenden Kurve dargestellt. Der Aktivierungseffekt ist in diesem Falle beim nichtoptimalen p_H von 3,3 relativ größer als beim optimalen p_H von 3,8. Nach der alkalischen Seite hin erfolgt dann ein rascher Abfall der relativen Aktivierungsgröße.

C. Über den zeitlichen Verlauf der autolytischen Reaktion

Der zeitliche Verlauf der Extraktautolyse wurde am Beispiel des Kalbsmilzauszuges verfolgt und der Effekt von Ferro- und Quecksilberionen untersucht.

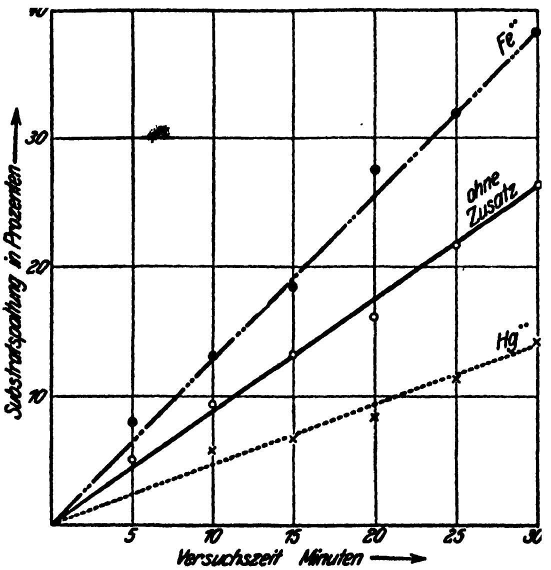


ABB. 8. Anfangsbereich der Kalbsmilzextraktautolyse ohne und mit Zusatz von Eisen und Quecksilbersalz.

Aus Abb. 8 ist ersichtlich, daß die Spaltkurve mit und ohne Zusätzen im Anfangsbereich der Spaltung innerhalb der Versuchsfehler linear verläuft. Der durchschnittliche Proportionalitätsfaktor für die unbeeinflußte Reaktion beträgt hier 0,88, derjenige für die durch Eisen beschleunigte Spaltung 1,34 und für die durch Quecksilber gehemmte Reaktion 0,46.

Beim Verfolgen der Spaltung über noch längere Zeit werden Kurven der in Abb. 9 dargestellten Form erhalten. Die Umsatzgeschwindig-

keit nimmt danach derart ab, daß die einzelnen Kurven einem asymptotischen Wert zuzustreben scheinen, derfür die unbeeinflußte, gehemmte und beschleunigte Reaktion ganz verschieden ist.

Bei Anwendung der Formel für monomolekularen Reaktionsverlauf ergibt sich gemäß der folgenden Zusammenstellung für die unbeeinflußte Reaktion von Anfang an ein kontinuierlicher Abfall der Reaktionskonstanten erster Ordnung. Das gleiche ist für den Ablauf unter

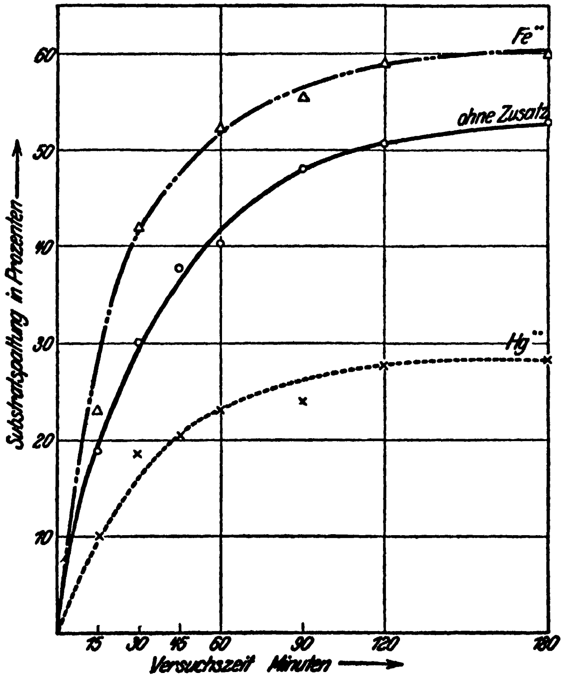


ABB. 9. Verlauf der Kalbsmilzextraktautolyse ohne und mit Eisen- und Quecksilberzusatz bei längerer Versuchszeit als der in Abb. 8 gewählten.

Einfluß von Quecksilber der Fall, während die durch Eisenzusatz beschleunigte Reaktion bis zu einem Substratumsatz von 52% gut als Reaktion erster Ordnung beschrieben werden kann. Im weiteren Verlauf fallen auch hier die Konstanten rasch ab. Der Verlauf der autolytischen Spaltkurve stimmt danach überein mit dem von *Rona* und *Kleinmann* ebenfalls auf nephelometrischem Wege studierten Abbau von Serumprotein durch Pepsin.

Die Frage nach der Beeinflussung der autolytischen Reaktion durch zugesetzte Spaltprodukte, sowie durch variierende Mengen hemmender und aktivierender Zusätze, ferner nach der Möglichkeit einer Affinitätsablenkung des Kathepsins vom Eigeneiweiß durch Zusatz von *Casein* befindet sich in Bearbeitung.

Zusammenstellung.

Anwendung der Formel $k = \frac{l}{t} \cdot \log \frac{a}{a-x}$ auf den Verlauf der Kalbsmilzextraktautolyse ohne und mit Zusatz von Eisen und Quecksilbersalz.
a = ursprüngliche Substratkonzentration = 100%,
x = die zur Zeit t abgebaute Substratmenge.

Ohne Zusatz			Eisen: $9 \cdot 10^{-3}$ mg/ccm			Quecksilber: $14 \cdot 10^{-3}$ mg/ccm		
Zeit Min.	Umsatz %	k	Zeit Min.	Umsatz %	k	Zeit Min.	Umsatz %	k
15	18,7	59	15	22,8	74	15	10	30
30	30	52	30	41,7	77	30	18,4	29
45	37,7	45	45	52,4	72	45	20,3	21
60	22,8	37	60	(52,3)	—	60	22,8	18
90	47,9	31	90	55	38	90	33,8	19
120	50,5	25	120	58,9	32	120	37,5	17
180	52,4	18	180	59,7	22	180	38,1	12

D. *Labwirkung der Organauszüge*

Im Hinblick auf die verschiedenartigen Angaben der Literatur über das Vorkommen von Labferment in den Geweben, bzw. über Labwirkung der Organproteasen selbst, wurden die verschiedenen Organauszüge in ihrem Verhalten gegen verdünnte und mittels Calciumzusatz sensibilisierte Milch geprüft. Dabei wurden, wie aus Tabelle III hervorgeht, Regelmäßigkeiten nicht beobachtet. In vielen Fällen unterschieden sich die Auszüge von verschiedenen Organen der gleichen Tiere voneinander; von den Cori-Rattentumorextrakten wirkte der eine labend, der andere nicht. Das gleiche war bei verschiedenen Kaninchennierenextrakten der Fall, bei denen allerdings infolge der Beimengung von Pepsinase eine deutliche Labwirkung zu erwarten gewesen wäre.

III. EXPERIMENTELLER TEIL

A. *Gewinnung der Gewebsextrakte*

Bei der Herstellung der Organextrakte wurde nach der von *Waldschmidt-Leitz* und *Deutsch* gegebenen Vorschrift verfahren:

Die sogleich nach dem Tode der Tiere entnommenen Organe wurden mechanisch von anhaftendem Gewebe befreit, mit Leitungswasser abgespült und sodann durch die Fleischmaschine getrieben oder (bei kleineren Organen) im Mörser mit dem Pistill zerkleinert. Der erhaltene Gewebsbrei wurde sodann in der doppelten Gewichtsmenge 87%igen Glycerins, dem 0,15% Essigsäure zugesetzt wurde, verteilt und im Brutschrank bei 37° C 4 Stunden lang unter gelegentlichem Umrühren digeriert. Nach Verlauf dieser Zeit wurde die Mischung auf Faltenfilter

TABELLE III

Übersicht über die Labwirkung von Organextrakten

Anordnung: 5 ccm pasteurisierter Milch + 5 ccm Aqua dest. + 4 Tropfen m-$CaCl_2$-Lösung + 30 Tropfen Organauszug, gemischt und im konstanten Temperaturraum bei 30° C belassen.

+++ Gerinnung innerhalb 30 Minuten,
++ " " 120 "
+ " " 17 Stunden.

Nr.	Extraktbezeichnung	Ergebnis	Nr.	Extraktbezeichnung	Ergebnis
1	Erwachsenenmilz I	++	13	Kaninchenniere IV	−
2	Erwachsenenleber I	++	14	Rattenleber a	−
3	Kinderleber II	+	15	Rattenleber b	−
4	Pferdeniere I	−	16	Rattenmilz a	++
5	Pferdemilz I	+	17	Rattenmilz b	++
6	Kalbsniere A	−	18	Ratten-Coritumor II	−
7	Kalbsmilz A	−	19	Ratten-Coritumor III	+
8	Kalbsleber A	+	20	Hühnerleber I	−
9	Kalbsmuskel A	−	21	Froschleber I	+
10	Kaninchenmagen III	+++	22	Karpfenmilz I	++
11	Kaninchenleber A	−	23	Karpfenleber I	++
12	Kaninchenniere III	++	24	Kontrolle ohne Extrakt	−

verteilt und das innerhalb der folgenden 48 Stunden erhaltene Filtrat als Gewebsextrakt zu den Versuchen benutzt und im Kühlschrank aufbewahrt.

In einem Falle wurde ein an Blut armer Kaninchenleberextrakt in folgender Weise gewonnen:

Ein ausgewachsenes Kaninchen wurde unter Äthernarkose von der Aorta aus mit 3 Litern physiologischer Kochsalzlösung durchspült. Ausfluß: Carotis. Sodann wurde die Leber entnommen und in der gleichen Weise, wie oben beschrieben, zu Glycerinextrakt verarbeitet.

Der aus den bluthaltigen Organen erhaltene Extrakt enthielt im Durchschnitt 0,8% des Eiweißkörpers, der bei der verwendeten Trü-

bungsreaktion mittels Sulfosalicylsäure analytisch erfaßt wurde. Der aus der durchspülten Kaninchenleber erhaltene Extrakt enthielt 96% des Eiweißes des gewöhnlichen Extraktes. Die fehlenden 4% dürften die Menge des ausgespülten Serumalbumins darstellen.

Die gewonnenen Extrakte waren meist klar und rotgefärbt. In einigen Fällen (Hühnerleber, Froschleber) waren sie trübe, doch konnten sie nach geeigneter Verdünnung gut zur Untersuchung benutzt werden.

B. Technik der nephelometrischen Analyse

Die Technik der nephelometrischen Bestimmung der Wirksamkeit von Organproteinasen auf zugesetztes Casein, ferner Gelatine und Serumalbumin findet sich ausführlich in der ersten Mitteilung von *Kleinmann* und *Stern* über tierische Gewebsproteasen beschrieben.

Es war dort notwendig, durch Auswahl geeigneter Trübungsreaktionen bzw. durch starke Verdünnung der Organextrakte den Einfluß des Eigeneiweißes der Extrakte auszuschalten. In der vorliegenden Untersuchung aber wurde ausschließlich der Abbau dieses Extrakteiweißes durch die im gleichen Extrakt befindlichen Enzyme verfolgt. Zu diesem Zwecke mußte eine weit höhere Extraktkonzentration als früher in den Spaltansätzen gewählt werden. In den meisten Versuchen betrug die Konzentration der Spaltansätze an Gewebsextrakt 3,3 oder 2,5%, in manchen jedoch 5%. In dieser Verdünnung lieferte das Extrakteiweiß mit Sulfosalicylsäurelösung und Salzsäure in den von *Kleinmann* für Serumalbuminlösungen angegebenen Mengen eine im Nephelometer von *Kleinmann* (*Schmidt* und *Haensch*, Berlin S 42) gut meßbare Trübung, die sich innerhalb der erforderlichen Zeit (etwa 30 Minuten) als ausreichend stabil erwies. Eine empirische Eichkurve mit abnehmenden Extrakteiweißkonzentrationen ergab bis zu 30% des ursprünglichen Proteingehalts herab befriedigende Proportionalität zwischen Konzentration und Trübung.

Während bei den früheren Untersuchungen infolge der geringen Enzymkonzentrationen Versuchszeiten von durchschnittlich 24 Stunden benötigt wurden, um deutliche Ausschläge zu erhalten, verläuft unter den hier gewählten Versuchsbedingungen die Spaltung so rasch, daß bereits innerhalb der ersten 100 Minuten nach Versuchsbeginn meist über 50% des Substrates zu Produkten abgebaut sind, die keine

Trübungsreaktion mit Sulfosalicylsäure liefern. Es muß daher in einem Wasserthermostaten mit gut regulierter Temperatur gearbeitet und die Trübungsreagenzien müssen möglichst umgehend nach der Probenentnahme zugegeben werden, um ein Fortschreiten des Prozesses zu verhindern. Bei sehr kurzen Spaltzeiten, wie sie z. B. bei der Ermittlung der Anfangsspaltgeschwindigkeiten nötig sind, muß die Enzymwirkung durch Einfließenlassen der Probe in eine abgemessene Menge schwacher Lauge momentan unterbrochen werden.

1. Technik für die Ermittlung der optimalen p_H-Aktivität

Zur Ermittlung der p_H-Aktivität wurde der *Veronalacetat-Salzsäure*puffer nach *Michaelis* benutzt. Ein Vergleich mit gewöhnlichem Acetatpuffer im Falle des Kalbsmilzkathepsins zeigte, daß p_H-Optimum dieses Enzyms gegenüber dem Extrakteiweiß bei der gleichen Azidität, nämlich bei p_H 4, gelegen ist, mit anderen Worten, daß das Veronal hier keinen spezifischen Effekt besitzt, sondern lediglich Pufferwirkung ausübt.

In eine Reihe von Meßzylindern (Volumen 75 ccm) werden 5 ccm einer m/7 Veronalnatrium-Natriumacetatlösung (9,714 g $C_2H_3O_2 \cdot Na \cdot 3H_2O$ + 14,714 g Veronalnatrium in 500 ccm Aqua dest.) sowie variierende Mengen n/10 Salzsäure (0 bis 25 ccm) zur Einstellung der gewünschten Azidität pipettiert. Sodann wird mit Aqua dest. auf 46 ccm aufgefüllt. Nach Vermischen werden mit der Pipette 10 ccm entnommen, je 4,5 ccm in zwei Reagenzgläser gegeben, der restliche Kubikzentimeter wird verworfen. Zu den im Zylinder verbliebenen 36 ccm werden 4 ccm der ausprobierten Extraktverdünnung (meist 1 : 3 oder 1 : 4) gegeben, bei längeren Spaltzeiten (über 2 Stunden) als Antiseptikum etwas Toluol zugefügt; nunmehr wird nach Verschluß mit einem Kautschukstopfen der Zylinder in den Wasserthermostaten bei 36° C eingehängt.

Nach Verlauf von 45 oder 60 Minuten werden 10 ccm des Spaltansatzes entnommen, je 5 ccm in zwei Reagenzgläser gegeben und anschließend zu den beiden Nullabnahmen von 4,5 ccm je 0,5 ccm der benutzten Extraktverdünnung gegeben. Sodann werden in alle vier Gläser je 10 ccm einer 12,5%igen Salzsäure + 7 ccm einer 20%igen Sulfosalicylsäurelösung pipettiert, gemischt und die beiden Spaltabnahmen und die eine der Nullabnahmen gegen die andere Nullabnahme als Standard im Nephelometer nach *Kleinmann* verglichen. Falls der Gehalt der zu untersuchenden Lösung an Organeiweiß zu niedrig sein sollte, um in der beschriebenen Anordnung mit HCl + Sulfosalicylsäure eine gut meßbare Trübung zu liefern (unter 1 mg pro 5 ccm), so kann die Trübungsintensität dadurch verstärkt werden, daß zu den Proben von 5 ccm Volumen 11 ccm Wasser + 4 ccm Sulfosalicylsäurelösung gegeben werden. Die durch die Sulfosalicylsäure *allein* erzeugte Trübung

ist unter Voraussetzung der niedrigen Eiweißkonzentration ausreichend stabil und besser sichtbar als die mittels Salzsäure + Sulfosalicylsäure erzeugte. Der Eiweißgehalt der Standardnullabnahme wird als 100% gewertet und die Abnahme der Konzentration in der Spaltabnahmen (berechnet nach dem *Beer*schen Gesetz) als fermentativer Substratabbau bezeichnet. Die Vornahme der nephelometrischen Bestimmung wird zweckmäßigerweise innerhalb der ersten 10 Minuten nach der Erzeugung der Trübung durchgeführt. (In den zur Analyse entnommenen Proben befanden sich 1 bis 2 mg Eiweiß.)

Im übrigen gelten die von *Kleinmann* für die Handhabung des Nephelometers ausgearbeiteten Richtlinien.

Um die p_H-Aktivität nach kürzerer und längerer Spaltzeit zu messen, kann entweder derart verfahren werden, daß z. B. eine Abnahme aus dem Spaltansatz nach 45 Minuten entnommen wird, worauf die Spaltung in diesen Proben unter Benutzung nur einer der beiden Nullabnahmen bestimmt wird, während die andere Nullabnahme dazu dient, bei einer späteren Spaltentnahme, nach z. B. 90 Minuten, als Standard zu fungieren, oder aber es werden den ursprünglichen 46 ccm Puffermischung anstatt 10 ccm 19,9 ccm für vier Nullabnahmen zu je 4,5 ccm entnommen. Zu den restlichen 26,1 ccm werden dann 2,9 ccm der Extraktverdünnung gegeben und nach Verlauf der gewählten Bestimmungsfristen immer je zwei Spaltabnahmen gegen zwei der Nullabnahmen analysiert.

In dem nach Beendigung des Versuchs verbliebenen Spaltansatzrest wird die Wasserstoffionenkonzentration in üblicher Weise elektrometrisch unter Benutzung der U-Elektrode mit stehender Wasserstoffblase ermittelt (siehe *Michaelis-Rona*, Praktikum der physikalischen Chemie, 4. Aufl., 1930, S. 192).

2. *Technik der Bestimmung des Einflusses verschiedener Zusätze*

In der vorliegenden Arbeit wurde der Einfluß verschiedener Zusätze, vornehmlich Schwermetalle, auf die Extraktautolyse meist im optimalen p_H-Gebiet des untersuchten Kathepsins (Pepsins oder Trypsins) studiert. Da bis auf den Fall der Tryptase die Optima im sauren Gebiet lagen, wurde bei den Versuchen mit Zusatzstoffen· die gewünschte Azidität mit Hilfe von m/15 *Acetat*puffern hergestellt, um einen etwaigen Einfluß des Veronalnatriums auszuschalten.

Zu 30 ccm des optimalen Acetatpuffergemisches (bereitet durch Vermischen von m/15 Essigsäure und m/15 Natriumacetatlösung in variierenden Verhältnissen) wird bei jedem untersuchten Enzym die gleiche Menge (meist 1 ccm der 1%igen) Metallsalzlösung zugefügt und sodann mit Aqua dest. auf 46 ccm Volumen aufgefüllt. Nach Vermischen werden 19,9 ccm für vier Nullabnahmen à 4,5 ccm entnommen und zu den restlichen 26,1 ccm 2,9 ccm der Extraktverdünnung pipettiert. Weiter wurde dann wie unter 1. beschrieben verfahren. War die Abnahme von Proben nach vier verschiedenen Spaltzeiten erwünscht, so wurde

unter Verzicht auf die Bestimmungsparallelen immer je eine Spaltentnahme gegen eine der vier Nullabnahmen analysiert.

Die aus Zweckmäßigkeitsgründen eingehaltene Konzentration an den verschiedenen Zusätzen betrug für *Kupfer* $7 \cdot 10^{-2}$ mg/ccm des Spaltgemisches (= 1 ccm einer 1%igen $CuSO_4$-Lösung); für *Zink* $8 \cdot 10^{-2}$ mg/ccm (= 1 ccm einer 1%igen $ZnSO_4$-Lösung); für *Quecksilber* $14 \cdot 10^{-2}$ mg/ccm (= 1 ccm einer 1%igen $HgCl_2$-Lösung); für *Mangan* $10{,}7 \cdot 10^{-2}$ mg/ccm (= 1 ccm einer m/10 $MnSO_4$-Lösung); für *Eisen* $7 \cdot 10^{-2}$ mg/ccm (= 1 ccm einer 1%igen $FeSO_4$-Lösung); für *Selen* $25 \cdot 10^{-2}$ mg/ccm (= 1 ccm einer 0,5%igen Na_2SeO_3-Lösung). Alle Angaben beziehen sich auf wasserfreie Salze.

Die erhaltenen Hemmungen und Aktivierungen sind wegen der nicht durchweg gleichen Konzentrationen nicht alle untereinander vergleichbar. Doch sind die korrespondierenden Werte für die verschiedenen untersuchten Enzyme untereinander vergleichbar, da die einmal gewählte Konzentration des Zusatzkörpers bei allen Enzymen die gleiche war.

Unter den Versuchsbedingungen (d. h. meist p_H 4) wurde durch die gewählten Zusatzkonzentrationen weder eine Farbänderung noch eine Trübung der Spaltansätze bewirkt.

Der nach Beendigung der Versuche unter Benutzung eines passenden Indikators (für p_H 4: Brom-Phenolblau) vorgenommene Vergleich der Spaltansätze ergab stets p_H-Gleichheit der Ansätze mit den und ohne die verschiedenen Zusätze, was ja infolge der guten Pufferung und der kleinen Konzentrationen der Zusatzstoffe auch zu erwarten war. *Die gefundenen Effekte sind also nicht etwa auf p_H-Verschiebungen zurückführbar.* Abb. 10 gibt ein Beispiel für die Reproduzierbarkeit der Metalleffekte.

3. Technik der kinetischen Versuche

Wenn die Spaltung über eine längere Zeit mittels mehr als vier Probeentnahmen verfolgt werden soll, so genügt es, alle obigen Angaben für die Zusammensetzung der Spaltmischungen zu verdoppeln oder eventuell zu verdreifachen, worauf Bestimmungen des Abbaues des Substrats nach acht bzw. zwölf verschiedenen Zeiten ausgeführt werden können. Soll jedoch die *Anfangsgeschwindigkeit* der Reaktion durch häufige Spaltabnahmen in kurzen Zeitintervallen (5 Minuten) ermittelt werden, so muß die Spaltung durch Einpipettieren der Proben in schwache Natronlauge (bei Spaltung bei p_H 4: n/20 NaOH) momentan unterbrochen werden.

Soll z. B. die Spaltung innerhalb der ersten 35 Minuten nach Versuchsbeginn in Intervallen von je 5 Minuten verfolgt werden, so werden wie üblich zwei Nullabnahmen à 4,5 ccm genommen. Sodann wird eine Reihe von Reagenzgläsern vorbereitet, indem in jedes Glas je 5 ccm der n/20 Natronlauge pipettiert werden.

Auch zu den Nullabnahmen werden je 5 ccm Lauge gegeben. Das Puffergemisch im Volumen von 36 ccm im Zylinder wird etwa 15 Minuten im Wasserthermostaten bei der Versuchstemperatur (z. B. 36° C) vorgewärmt, worauf dann unter Markierung der Zeit 4 ccm der Extraktverdünnung zugefügt werden. Nach Ablauf von 5 Minuten werden aus der Mischung 5 ccm entnommen und sogleich in eins der mit Lauge beschickten Gläser pipettiert. Dies wird nach weiteren fünf Minuten und

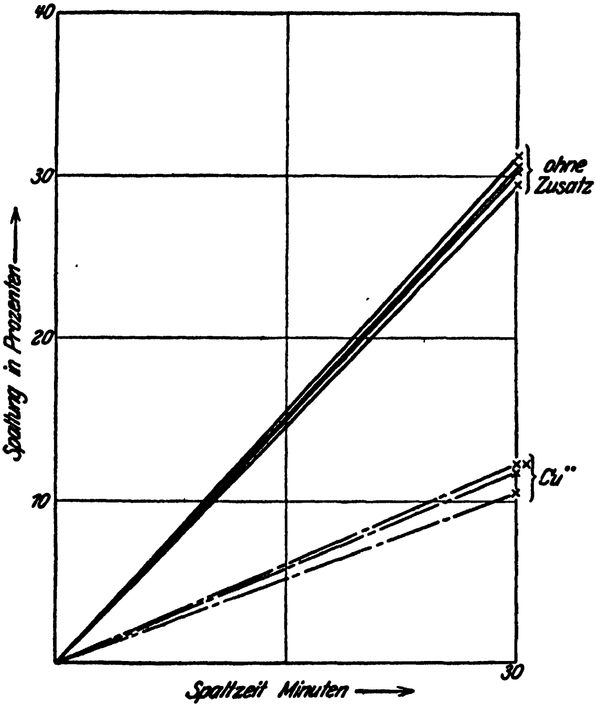


ABB. 10. Beispiel für die Reproduzierbarkeit eines Effektes auf die Extraktautolyse: Hemmung der Froschleberextraktautolyse durch Kupfer in einer Konzentration von $7 \cdot 10^{-2}$ mg Cu pro ccm. Je vier Parallelversuche mit und ohne Kupferzusatz.

so fort wiederholt, bis sieben Proben entnommen worden sind. (In den im Zylinder verbliebenen restlichen 5 ccm kann bequem p_H gemessen werden.) Sodann werden zu den beiden Nullabnahmen je 0,5 ccm des Extrakts gegeben und in alle Gläser je 5 ccm einer 25%igen Salzsäure und weiter 7 ccm der 20%igen Sulfosalicylsäure pipettiert. Innerhalb der folgenden 10 Minuten werden die Trübungen der Spaltentnahmen im Nephelometer gegen die eine Nullabnahme als Standard gemessen, wobei die zweite Nullabnahme als Kontrolle des Standards dient.

Werden mehrere derartiger kinetischer Versuche, z. B. mit variierenden Metall-

mengen, gleichzeitig angestellt, so muß darauf geachtet werden, daß die Abnahme der einzelnen Proben exakt in gleichen Zeitintervallen nacheinander erfolgt, da sonst in Anbetracht der kurzen Versuchsintervalle relativ große Fehler entstehen können.

C. Auswertung der Versuchsergebnisse

Bei der Konstruktion der *Aktivitäts-p_H-Kurven* wird folgendermaßen verfahren:

Als Abszisse werden die elektrometrisch gemessenen p_H-Werte, als Ordinate die berechneten Werte der Substratspaltung in Prozenten aufgetragen. Die Spaltung in Prozenten wird erhalten, indem die nach der betreffenden Versuchszeit nephelometrisch ermittelte Substratkonzentration von 100 subtrahiert wird.

Bei der Berechnung der gefundenen *Hemmungs- und Aktivierungseffekte* zugesetzter Substanzen (siehe Tabelle II) wird derart vorgegangen, daß die durch die unbeeinflußte Protease bewirkte Substratspaltung gleich 100% Aktivität gesetzt wird. Nunmehr wird die in entsprechenden Intervallen nach Zusatz eines Salzes gefundene höhere bzw. geringere Substratspaltung gegen die Spaltung des unbeeinflußten Enzymsubstratgemisches im gleichen Zeitpunkt verglichen und die Aktivität des derart beeinflußten Enzyms in Prozenten der Aktivität des ungehemmten Enzyms ausgedrückt.

Wird beispielsweise gefunden, daß die in gleichen Zeitintervallen bestimmten Abbauwerte für einen Extrakt nach Zugabe von Eisensalz durchschnittlich doppelt so groß wie die korrespondierenden Werte des unbeeinflußten Enzyms sind, so wird unter Gleichsetzung der Aktivität mit dem in der Zeit erzielten Umsatz die Aktivität des betreffenden Enzyms unter Eisenzusatz zu 200% angegeben.

Die Auswertung der *kinetischen Versuche* erfolgt unter Zuhilfenahme der bekannten Beziehungen zwischen Zeit und Umsatz, wobei der Nachteil mit in Kauf genommen werden muß, daß die Natur des Untersuchungsobjektes es nicht zuläßt, das Verhältnis der Substratzu der Fermentmenge zu variieren.

D. Typische Versuchsprotokolle

Auf eine Wiedergabe der gesamten, in den Tabellen aufgeführten Versuche in Protokollform muß naturgemäß verzichtet werden. Nur

einige wenige Protokolle seien zur Veranschaulichung der gewählten Anordnungen angeführt:

BEISPIEL NR. 1

Ermittlung der Aktivitäts-p_H-Kurve der Kalbsmilzextraktspaltung (Vers. 33)

Anordnung: In acht Meßzylinder (Volumen 50 ccm) werden je 5 ccm der m/7 Veronal-Acetatmischung sowie die unten angegebenen Mengen n/10 HCl pipettiert. Auffüllen mit Aqua dest. auf 46 ccm Volumen, Entnahme von 10 ccm für zwei Nullabnahmen à 4,5 ccm. Zu den restlichen 36 ccm werden 4 ccm des mit Aqua dest. auf das Dreifache verdünnten Kalbsmilzextrakts A pipettiert und die Zylinder in den Wasserthermostaten von 36° C eingehängt. Die Extraktverdünnung, die später zu den Nullabnahmen gegeben werden muß, wird inzwischen im Kühlschrank aufbewahrt. Nach Ablauf von 120 Minuten werden jedem der Spaltansätze 10 ccm entnommen, zweimal je 5 ccm davon in ein Reagenzglas pipettiert. Zu den Nullabnahmen werden je 0,5 ccm der Extraktverdünnung gegeben und nunmehr zu allen Gläsern je 10 ccm 12,5%iger HCl + 7 ccm 20%iger Sulfosalicylsäurelösung pipettiert. Messung nach 3 Minuten im *Kleinmann*schen Nephelometer (*Schmidt* und *Haensch*, Berlin).

Ergebnis:

Ansatz-Nr.	ccm n/10 HCl	p_H	Spaltung nach 120 Min. %
1	19	2,72	20
2	15,4	3,95	52
3	12,4	4,66	30,6
4	9,0	5,41	5
5	7,1	6,76	0
6	6,0	7,09	0
7	2,6	7,82	0
8	10 ccm n/100 NaOH	9,52	0

Das optimale p_H ist demnach 4.

BEISPIEL NR. 2

Aktivitäts-p_H-Kurve der Karpfenleberextraktspaltung (Versuch 51)

Anordnung: Die gleiche wie in Beispiel Nr. 1, nur an Stelle des Kalbsmilzextrakts wurde der dreifach verdünnte *Karpfenleberextrakt 1* benutzt. Vornahme der Spaltung bei 43°.

Ansatz-Nr.	ccm Säure oder Lauge		p_H	Spaltung nach	
				15 Min. %	45 Min. %
1	20	ccm n/10 HCl	2,05	4	14,9
2	15,4		2,57	20	28,6
3	12,4		4,08	31	50
4	9		4,88	24,3	35,5
5	7		5,65	26,8	38,5
6	4		7,41	31	48,4
7	0		8,55	41,2	55,1
8	10	ccm n/100 NaOH	9,09	47,4	56,2
9	10		9,18	46,7	55,9
10	20		9,94	53,5	61,1
11	30		10,69	46,7	44,1
12	1 ccm n NaOH		11,7	5	18,4

Die Karpfenleberextraktautolyse weist demnach zwei ausgeprägte Optima auf, eines im schwach sauren Gebiet bei p_H 4 (Kathepsin) und eines im alkalischen Gebiet bei p_H 10, das dem von den Pankreasinseln sezernierten Trypsin zukommen dürfte.

BEISPIEL NR. 3

Effekt von Kupfer-, Zink-, Quecksilber-, Ferro-, Mangan- und Selenitionen auf die Hühnerleberextraktspaltung (Versuch 26)

Anordnung: In sieben Meßzylinder (50 ccm Inhalt) wurden 15 ccm eines m/15 Acetatpuffers 4/1 (p_H 4,1) gegeben. Sodann wurden bis auf Zylinder Nr. 1 die unten aufgeführten Salzlösungen zugefügt und mit Aqua dest. auf 46 ccm aufgefüllt. Entnommen 19,9 ccm für vier Nullabnahmen à 4,5 ccm. Zu den restlichen 26,1 ccm wurden je 2,9 ccm des dreifach verdünnten Hühnerleberextrakts I pipettiert. Analyse im Nephelometer wie gewöhnlich. Das p_H der Ansätze wurde nach Beendigung des Versuchs mittels Vergleich mit Brom-Phenolblau als gleich befunden.

Ansatz-Nr.	Zusatz	Spaltung nach		
		60 Min. %	120 Min. %	180 Min. %
1	Kein Zusatz	24,3	36,3	41,4
2	1 ccm $CuSO_4$- Lösung, 1%ig	6,6	17,4	18,4
3	1 " $ZnSO_4$- " 1%ig	23	35,7	39,4
4	1 " $HgCl_2$- " 1%ig	7,9	8,3	9
5	1 " $MnSO_4$- " m/10	31	45,2	52,4
6	1 " $FeSO_4$- " 1%ig	30,6	42	46,7
7	4 " Na_2SeO_3- " 0,5%ig	4,8	13	13

Ergebnis. Wenn die Aktivität der unbeeinflußten Protease gleich 100% gesetzt wird, so betrug sie unter dem Einfluß von

$7 \cdot 10^{-2}$	mg $Cu^{\cdot\cdot}$	pro	Kubikzentimeter	36%
$8 \cdot 10^{-2}$	„ $Zn^{\cdot\cdot}$	„	„	100%
$14 \cdot 10^{-2}$	„ $Hg^{\cdot\cdot}$	„	„	26%
$10{,}7 \cdot 10^{-2}$	„ $Mn^{\cdot\cdot}$	„	„	127%
$7 \cdot 10^{-2}$	„ $Fe^{\cdot\cdot}$	„	„	118%
$25{,}0 \cdot 10^{-2}$	„ Se	„	„	28%

BEISPIEL NR. 4

Untersuchung der Kinetik des Anfangsbereiches der Spaltung des Kalbsmilzextrakts unter dem Einfluß von Eisen- und Quecksilberionen (Versuch 79)

Anordnung: In drei Meßzylinder (Volumen 50 ccm) wurden je 35 ccm m/15 Acetatpuffer 4/1 (p_H 4,1) sowie die betreffenden Salzlösungen gegeben und dann mit Aqua dest. auf 46 ccm aufgefüllt. Entnahme von 10 ccm für zwei Nullabnahmen zu je 4,5 ccm. Zu den restlichen 36 ccm werden nach Vorwärmen im Thermostaten bei 36° C je 4 ccm des dreifach verdünnten Kalbsmilzextrakts A pipettiert. Für jeden Zylinder werden sechs Reagenzgläser mit je 5 ccm n/20 NaOH vorbereitet. Auch zu den Nullabnahmen werden je 5 ccm Lauge gegeben. Abnahmen in Intervallen von 5 Minuten, alle Proben werden gemeinsam nephelometriert.

Ansatz-Nr.	Zusatz	Spaltung nach					
		5 Min. %	10 Min. %	15 Min. %	20 Min. %	25 Min. %	30 Min. %
1	Kein Zusatz	4,8	9,1	13	16	21,6	26,5
2	1 ccm $HgCl_2$-Lösung, 1%ig	4,8	5,7	6,6	8,2	11,2	13,8
3	1 „ $FeSO_4$- „ 1%ig	7,9	13	18,4	27,3	31,8	38,3

Ergebnis. Die graphische Darstellung (Abb. 8) zeigt, daß die drei Spaltkurven im untersuchten Gebiet innerhalb der experimentellen Fehlergrenzen *linear* verlaufen. Aktivierung durch Eisen, Hemmung durch Quecksilber.

IV. ZUSAMMENFASSUNG

1. Die erste Stufe des Abbaus gelöster Organproteine durch die in den gleichen Extrakten enthaltenen tierischen Gewebsproteinasen wurde auf *nephelometrischem* Wege studiert. Es wird ausführlich eine Methode beschrieben, die es gestattet, diese *autolytische* Reaktion in gleich kurzen Zeiträumen (30 bis 180 Minuten) und ebenso exakt

messend zu verfolgen, wie dies bisher nur bei *peptischen* und *tryptischen* Spaltungen möglich war.

2. Bis auf einige Sonderfälle, die durch die Anwesenheit sezernierter Proteasen des Verdauungstraktes erklärt werden konnten, wurde in den untersuchten Organauszügen (aus *Niere*, *Milz*, *Leber*) jeweils nur eine einzige echte Proteinase (Kathepsin) gefunden, die das Eigeneiweiß optimal zwischen p_H 3,2 und 4,5 angreift.

3. Der Effekt von $Cu^{\cdot\cdot}$-, $Hg^{\cdot\cdot}$-, $Zn^{\cdot\cdot}$-, $Fe^{\cdot\cdot}$-, $Mn^{\cdot\cdot}$- und SeO_3''-Ionen auf die beim optimalen p_H ablaufende autolytische Reaktion wurde untersucht und dabei ein verschiedenartiges Verhalten der Gewebsproteinasen beobachtet. Allgemein wirkte Eisen- und Manganzusatz aktivierend, Quecksilber, Kupfer und Selenit hemmend, Zink manchmal hemmend, manchmal aktivierend. Im Speziellen finden sich bei den Fermenten der verschiedenen Organe und verschiedenen Spezies quantitative und reproduzierbare Differenzen im Verhalten gegen die genannten Zusätze. Eine einheitliche Deutung der Metalleinflüsse wird besonders durch den Umstand erschwert, daß dieselben sich durchaus nicht immer im Sinne einer Hemmung, sondern in vielen Fällen auch in einer Aktivierung geltend machen. Die Unterschiede zwischen den Kathepsinen der untersuchten Spezies sind meist größer als diejenigen zwischen den Kathepsinen der verschiedenen Organe der gleichen Tierart.

4. Der Verlauf der Spaltung ist auf einer merklichen Strecke linear von der Zeit abhängig. Später flacht die Spaltkurve ab, und der Umsatz nähert sich einem asymptotischen Grenzwert, bevor das vorhandene Substrat aufgespalten ist. Die Höhe dieses Endwertes liegt bei Gegenwart hemmender Stoffe niedriger, und bei Zusatz fördernder Stoffe höher als im Kontrollversuch.

5. Eine Anzahl der gewonnenen Organauszüge zeigte gegen Milch Labwirkung.

Herrn Professor Dr. *Michaelis* sei auch an dieser Stelle aufs beste für seine vielfachen Ratschläge und für die Zurverfügungstellung eines Arbeitsplatzes gedankt. Herrn Geheimrat Dr. *C. Duisberg* danke ich bestens für die Ermöglichung der Untersuchung durch Gewährung eines Auslandsstipendiums.

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THE TITRATION OF HYDROXY ORGANIC ACIDS IN THE PRESENCE OF FERRIC AND CUPRIC SALTS

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In previous work (1, 2) it was pointed out from theoretical reasoning that when a hydroxy organic acid combines with a metal such as iron to form an unionized compound the acid properties of the alcoholic hydroxyl group must be increased. There is abundant evidence in the literature which indicates that the hydrogen of such hydroxyl groups may be replaced by a metal. References to most of this work may be found in the handbooks of Gmelin (3) and Abegg *et al.* (4). There is apparently no direct evidence, however, showing how strongly acidic these groups become. The only titration curves with which the writer is familiar are those of Wark and Wark (5) on the titration of the normal copper salts of several such acids with sodium hydroxide. These authors followed the potential of their solutions during the titration by means of a hydrogen electrode. It is apparent from the potential values that they obtained, that they were not measuring pH but an oxidation-reduction potential instead. The present paper is the result of an attempt to follow, by means of a glass electrode, the titration of representative hydroxy organic acids alone and in the presence of ferric chloride and of cupric chloride.

The glass membranes were made in the manner described by MacInnes and Dole (6). The glass used was obtained from the Corning Glass Company, their No. 015. The MacInnes type of permanently sealed electrode containing 0.1 M HCl and a Ag, AgCl wire was used. The membranes were calibrated against buffers of known pH and this calibration was checked at frequent intervals. The electromotive force was measured by means of a

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vacuum tube potentiometer arrangement of the type described by Partridge (7). The readings obtained were accurate to 1 millivolt.

The results are presented in Figs. 1 to 6. Curves are included for ferric chloride, for cupric chloride, for each of the following acids, lactic, glycollic, oxalic, malic, tartaric, and citric, and for each of

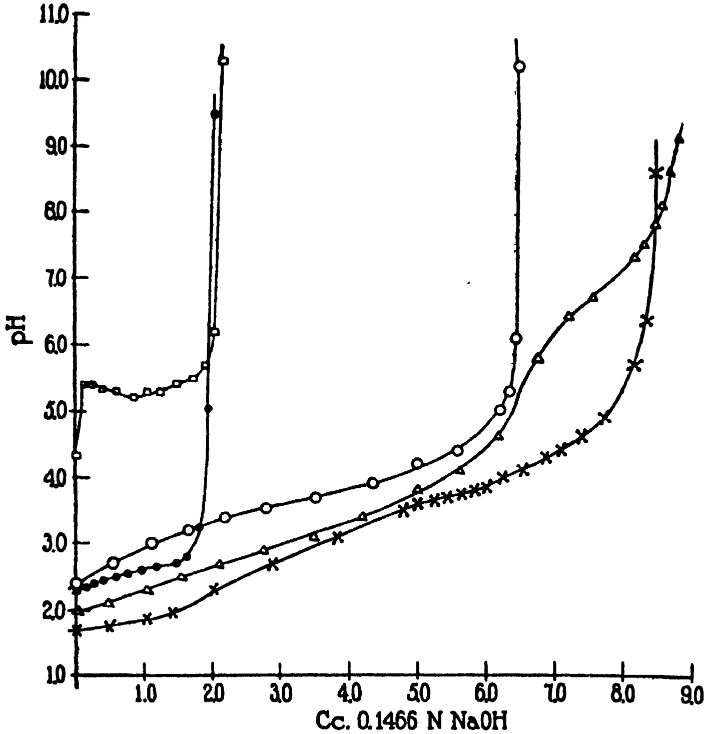


FIG. 1. □ indicates the curve for 1.0 cc. $CuCL_2$, ● 1.0 cc. $FeCl_3$, ○ 5.0 cc. lactic acid, △ 5.0 cc. lactic acid + 1.0 cc. $CuCl_2$, × 5.0 cc. lactic acid + 1.0 cc. $FeCl_3$.

these acids in the presence of ferric chloride and in the presence of cupric chloride.

It may be seen from each of the figures that the mixtures of acid and metal chloride are considerably more acidic than either of these substances alone. The beginning of each curve where iron is present clearly indicates that we are titrating some strong

acid. We interpret this as an indication of the fact that the metal and the acid combine to form a complex with the liberation of free hydrochloric acid.

Let us examine the results with lactic acid (Fig. 1) in some detail. It may be seen that the quantity of cupric chloride used requires 2.0 cc. of base for its neutralization. At this point all the copper has been precipitated as cupric hydroxide. The

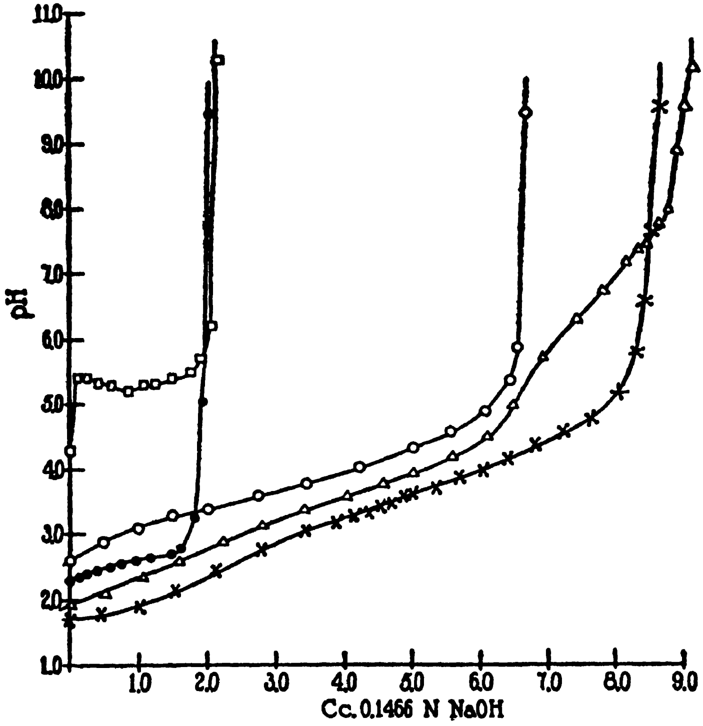


FIG. 2. □ indicates the curve for 1.0 cc. $CuCl_2$, ● 1.0 cc. $FeCl_3$, ○ 5.0 cc. glycollic acid, △ 5.0 cc. glycollic acid + 1.0 cc. $CuCl_2$, × 5.0 cc. glycollic acid + 1.0 cc. $FeCl_3$.

quantity of lactic acid used requires 6.5 cc. of base for its neutralization. Now, if the cupric chloride and lactic acid are mixed and the mixture is titrated it requires an amount of base equal to the sum required for the two separate solutions. At this point the copper has again been precipitated as cupric hydroxide. Thus, under the conditions of these experiments lactic acid is unable to prevent

the precipitation of copper as the hydroxide in a neutral or alkaline solution. If we add to the lactic acid an amount of ferric chloride equivalent to the cupric chloride previously added we get a quite different curve. Again the end-point comes when an amount of base equivalent to the two separate solutions has been added, but at this point there is no precipitation of ferric hydroxide. It

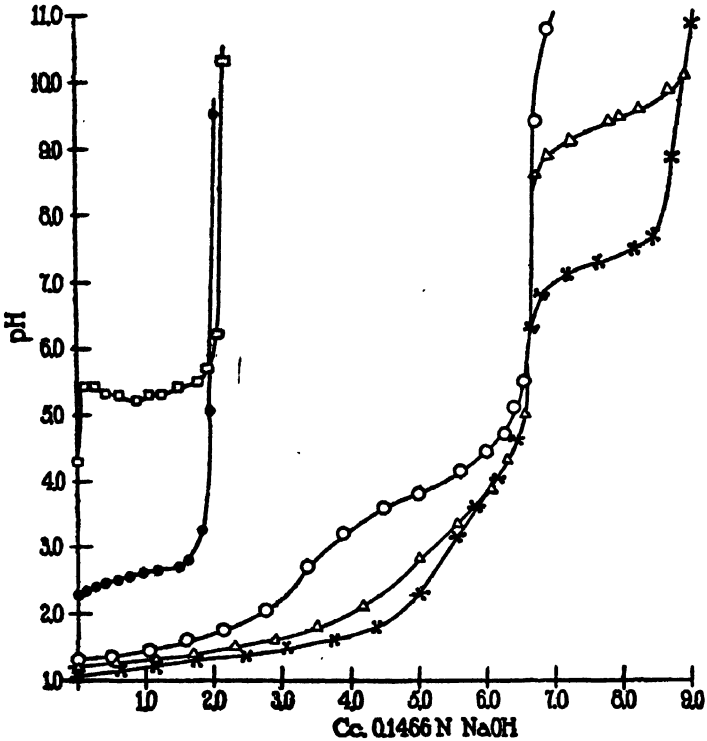


FIG. 3. □ indicates the curve for 1.0 cc. $CuCl_2$, ● 1.0 cc. $FeCl_3$, ○ 5.0 cc. oxalic acid, △ 5.0 cc. oxalic acid + 1.0 cc. $CuCl_2$, × 5.0 cc. oxalic acid + 1.0 cc. $FeCl_3$.

should be noticed that there is a distinct break in this curve at a point corresponding to pH 3.5. The significance of this break will be discussed later.

The results with glycollic acid are presented in Fig. 2. In general they are the same as those with lactic acid. The break in the curve for acid plus iron is less marked but still definitely perceptible.

It may be well to examine, at this point, the curves obtained with some acid that forms a complex with metals, but which does not possess an alcoholic hydroxyl group. Such an acid is oxalic. Fig. 3 presents the curves obtained with it. It may be seen that the first end-point occurs at the end-point of the oxalic acid itself and this is followed by the precipitation of the ferric or cupric

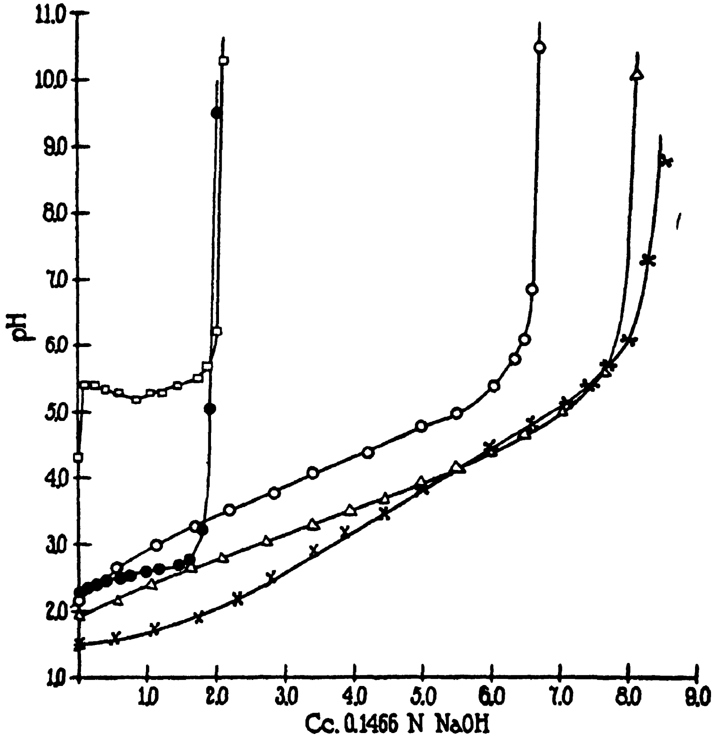


FIG. 4. □ indicates the curve for 1.0 cc. $CuCl_2$, ● 1.0 cc. $FeCl_3$, ○ 5.0 cc. malic acid, △ 5.0 cc. malic acid + 1.0 cc. $CuCl_2$, × 5.0 cc. malic acid + 1.0 cc. $FeCl_3$.

hydroxide. Under the conditions of these experiments this acid does not prevent the precipitation of either ferric or cupric hydroxide in an alkaline solution. An important point to be observed is that that part of the curve which corresponds to the second constant of the oxalic acid lies in a much more acid range when either iron or copper is present than in their absence.

Figs. 4 to 6 present the results obtained with malic, tartaric, and citric acids, respectively. It may be observed that with these acids the behavior of iron and of copper is similar. In each case the amount of base required to titrate a mixture of acid plus metal chloride is very considerably more than that required for the acid alone and in no case is there any precipitation of metal

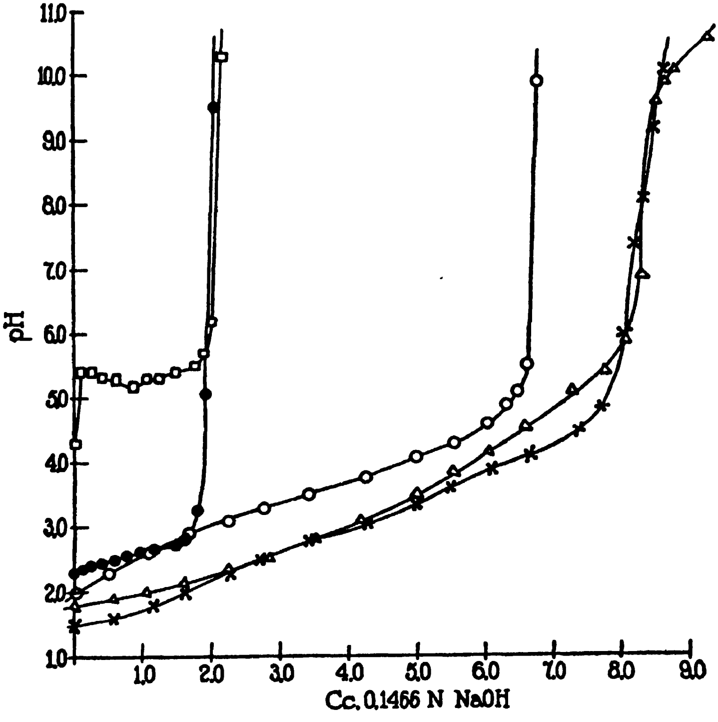


FIG. 5. □ indicates the curve for 1.0 cc. $CuCl_2$, ● 1.0 cc. $FeCl_3$, ○ 5.0 cc. tartaric acid, △ 5.0 cc. tartaric acid + 1.0 cc. $CuCl_2$, × 5.0 cc. tartaric acid + 1.0 cc. $FeCl_3$.

hydroxide. It should also be noted that in no case does the titration of such a mixture require an amount of base as large as the sum required for the titration of the two solutions separately.

DISCUSSION

Let us consider the results with lactic acid first. We saw that the mixture of lactic acid and ferric chloride required for its

neutralization an amount of base equal to that required for the two solutions separately. When the solutions are titrated separately all of the iron is precipitated as ferric hydroxide, but when the mixture is titrated no precipitate is formed. The question is, how did we use up so much base without precipitating any ferric hydroxide. There are at least two possible answers. The

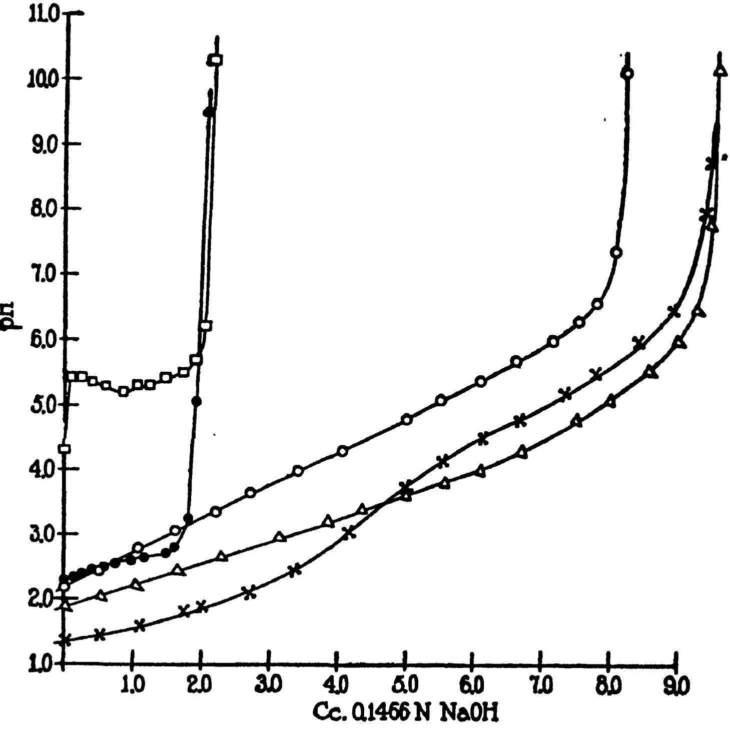


FIG. 6. □ indicates the curve for 1.0 cc. $CuCl_2$, ● 1.0 cc. $FeCl_3$, ○ 5.0 cc. citric acid, △ 5.0 cc. citric acid + 1.0 cc. $CuCl_2$, × 5.0 cc. citric acid + 1.0 cc. $FeCl_3$.

first of these is that ferric hydroxide was really formed, but it remained in colloidal solution instead of precipitating. One fact seemingly in favor of this view is that these solutions are not readily diffusible through a collodion bag. However, if one examines the various compounds reported by Gmelin (3) another explanation suggests itself for this lack of diffusibility. This is that the compounds which exist in solution have high molecular

weights. Many of those reported contain 3 or more iron atoms and a corresponding number of organic residues. We have tested the diffusibility of the alkaline solutions of iron with each of the acids studied. In each case it is very slow, but in each case some iron passes through the collodion bag, in contrast to the complete lack of diffusibility with an ordinary sol of ferric hydroxide.

There are other difficulties in the way of accepting this colloidal explanation. These solutions are not sensitive to electrolytes. Any one of them may be saturated with ammonium sulfate without producing any precipitate. Relatively large amounts of calcium chloride do produce a precipitate, but this precipitate is not ferric hydroxide. It would seem to be the calcium salt, analogous, in the case of lactic acid, to the sodium, potassium, and ammonium salts isolated by Hofmann (8) to which he assigned the formula $M[Fe(CH_3CHOCO_2)_2]$, where M represents either sodium, potassium, or ammonium.

If these solutions contain the iron as colloidal ferric hydroxide then we have the problem of determining why an alcoholic hydroxyl group is so very important for the maintenance of the colloidal state. Thus lactic acid and glycollic acid would be good peptizing agents whereas propionic acid and acetic acid are of no avail. Similarly, neither oxalic acid nor succinic acid can produce the necessary action, but malic acid works very nicely and tartaric acid is exceedingly effective.

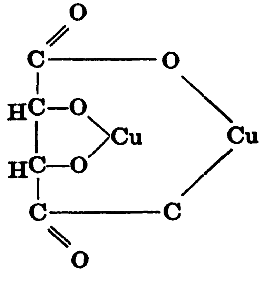
The other explanation is that when the lactic acid and iron combined to form a complex the alcoholic hydroxyl group took part in this combination. Such an action must make the oxygen of this group more positive and as a result the hydrogen must become more acidic. Then to the extent that iron is present lactic acid will behave as a dibasic acid. This will account nicely for the amount of base used without making it necessary to assume the formation of ferric hydroxide. Furthermore, it will account nicely for the two steps in the titration curve for lactic acid plus ferric chloride. According to this interpretation the pK_a for this alcoholic hydroxyl group is about 3.85. We may point out that this interpretation is in accord with our previous contention (2), that in dehydrogenation reactions the hydrogen separates from the molecule as hydrogen ion and the process of activation is simply a process of increasing the acid dissociation constant of the group involved.

An interesting observation concerning the acidity of such hydroxyl groups has recently been reported by Hölzl (9). He found that if the ester of salicylic acid was dissolved in absolute alcohol the hydroxyl group then exhibited appreciably acid properties.

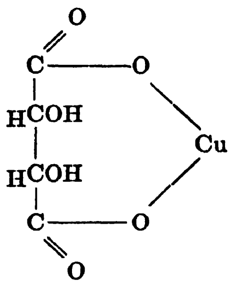
The explanation given for the results obtained with lactic acid will apply equally well to those obtained with glycollic acid. Let us see now how it will apply to oxalic acid. If this free acid forms an unionized compound with iron, and the curves (Fig. 3) show that it does, we would expect that the OH group of the undissociated carboxyl would take part in the combination. The result should be that this second carboxyl group would become a stronger acid than it is in the absence of a metal. The curves show that this is markedly true.

The same general explanation will apply to the results with malic, tartaric, and citric acids. Here we have the added complication, however, that the amount of base required for the neutralization of a mixture of any one of these acids with ferric or cupric chloride is not quite as large as the sum required for the neutralization of the two separate solutions. The explanation for this must lie in the formation of complex basic salts. This is in agreement with the results obtained by Packer and Wark (10) and by Wark and Wark (5) in their work on copper tartrates, as well as with the results of many other investigators (3, 4). Dumanskii and Chalisew (11), however, regard these copper tartrates as colloidal solutions of copper hydroxide. Similarly, Dumanskii and Yakovlev (12) regard solutions of ferric iron and various hydroxy acids as colloidal solutions of ferric hydroxide.

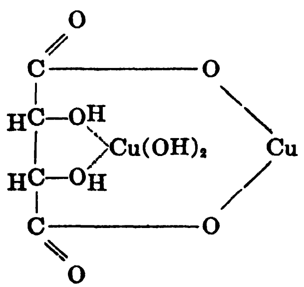
We may inquire here just what the difference is between the colloidal and the non-colloidal view-point regarding these solutions. Jellinek and Gordon (13) have obtained a crystalline copper salt of copper tartrate. They assigned to it this formula:



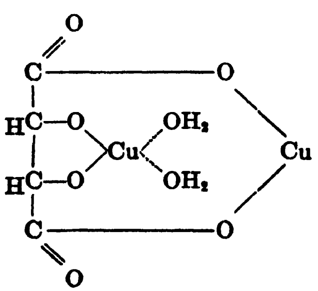
Dumanskii and Chalisew (11) have prepared what they believe is an identical salt and they assign to it this formula: Cu*T*·$Cu(OH)_2$·H_2O. The *T* indicates tartrate. Let us see how this differs from Jellinek and Gordon's formula. We may write the Cu*T* as



To this is attached a $Cu(OH)_2$ by means of a point (·). We must interpret this point as some kind of an attraction and this attraction must be furnished by the OH groups of the tartrate, for one cannot substitute succinic acid for the tartaric acid. By incorporating this into the formula we have



We may rewrite this as



This now differs from Jellinek and Gordon's formula by 2 molecules of water. If Jellinek and Gordon's compound be taken to represent the non-colloidal point of view and Dumanskii and Chalisew's compound to represent the colloidal point of view then, remembering that basic compounds may also be formed in which there is one copper attached to each hydroxyl group, and that one copper may be attached to a hydroxyl group in each of 2 tartrate molecules, there is really no difference as far as present data are concerned between the two views.

The application of these results to the titration of any one or any mixture of the hydroxy acids considered is clear. If one were to determine by titration the amount of these acids present in a solution containing proportionately as much iron, or in some cases as much copper, as the solutions used here, and were to use the turning point of phenolphthalein as end-point, the result would be in error by over 30 per cent.

SUMMARY

Titration curves, obtained by means of the glass electrode, are reported for lactic, glycollic, oxalic, malic, tartaric, and citric acids, and for each of these acids in the presence of ferric chloride and in the presence of cupric chloride.

It is shown that the presence of these metal salts greatly alters the titration curves. The nature of this change is discussed and the application of the results is pointed out.

It is a pleasure to acknowledge my indebtedness to Dr. L. Michaelis, in whose laboratory this work was carried out, for his generous assistance and interest in this work.

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STUDIEN ÜBER DAS LYMPHATISCHE GEWEBE MIT BESONDERER BERÜCKSICHTIGUNG DER LYMPHOPOËSE UND DER HISTOGENESE DER SEKUNDÄRKNÖTCHEN, IHRES SCHICKSALS UND IHRER BEDEUTUNG

(V. Mitteilung)

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(Eingegangen am 18. Oktober 1930)

EINLEITUNG

Die lymphatischen Organe, deren Bedeutung für die parenterale Verdauung hinreichend bekannt und Gegenstand vieler Untersuchungen gewesen ist, sind, wie wir wissen, überall in die Säfteströme als Filter für Stoffwechselprodukte und eindringende Fremdkörper eingeschaltet. Nichts spricht deutlicher dafür, als ihre regionäre Anordnung und ihre fast unausbleibliche Reaktion auf Reize in den vorgelagerten Bezirken. Eine weitere bekannte Funktion dieser Organe ist die der Lymphocytenproduktion. In ihnen werden, vielleicht in enger Beziehung zur parenteralen Verdauung, Lymphocyten gebildet, die teils durch die Lymphgefäße, teils direkt in die Blutbahn auswandern. Schließlich spielen sie eine wichtige Rolle bei der Bildung von Schutzstoffen. Sie scheinen die einzigen Orte zu sein, in welchen bisher eine sichere Antikörperbildung nachgewiesen werden konnte (Bezançon und Labbé (1898), Manfredi (1899), Manfredi und Viola (1899)). Dabei müssen wir uns wohl auch die Schutzstoffe als Stoffwechselprodukte einer parenteralen Verdauung vorstellen, und zwar der cellulären Elemente dieser Organe, welche entweder das Antigen selbst aufnehmen oder erst durch Zwischenprodukte von anderen Zellen zur Antikörperbildung angeregt werden (Ribbert (1907)).

Die Zellen, welche als Träger der parenteralen Verdauung in Frage kommen, sind die Lymphocyten und Reticuloendothelien, aus welchen die lymphatischen Gewebe zusammengesetzt sind und welche ihnen ihr

charakteristisches Gepräge geben. Außerdem sind diese Gewebe reich an undifferenzierten Mesenchymzellen, wie besonders MAXIMOW (1927) ausgeführt hat.

Die Lymphocyten teilt man am besten in große, mittelgroße und kleine Lymphocyten ein (Taf. XI, Abb. 5–7). Die mittelgroßen Lymphocyten sind die eigentlichen Keimzentrumszellen. Die großen Lymphocyten habe ich früher (1929 c) mit MARCHAND (1924) als proliferierende Reticuloendothelien bezeichnet. MARCHAND hat sie jedoch mit den Histiocyten verwechselt und ihnen die Fähigkeit der Speicherung und Phagocytose zugeschrieben. Da wohl auch die großen Lymphocyten schon mehr oder weniger einseitig ausdifferenzierte Zellen sind und keine prospektiven Potenzen mehr haben, wollen wir sie hier, wie es auch MAXIMOW (1927) getan hat, als große Lymphocyten bezeichnen.

Unter Reticuloendothelien verstehe ich hier ausschließlich ASCHOFF's reticuloendothelialen Apparat im engeren Sinne (1924), dessen Zellen noch im Verbande, aber schon mehr oder weniger einseitig ausdifferenziert sind. Sie sind verhältnismäßig große Zellen mit breitem meist acidophilen Protoplasma (Taf. XI, Abb. 8–9) und sind dadurch ausgezeichnet, daß sie retikuläre Fasern bilden und stark speichern können. Man kann diese Zellen nach topographischen Gesichtspunkten in Reticulum- und Uferzellen einteilen, je nach dem sie im lymphatischen Gewebe liegen oder Lymphsinus auskleiden. Ich möchte dabei SIEGMUND's Bezeichnung „Uferzellen“ dem Namen „Endothelien“ oder „eigentliche Reticuloendothelien“ vorziehen, da als Reticuloendothelien beide Zellen bezeichnet werden, und der Name „Endothelien“ für eine biologisch ganz anders charakterisierte Zellgruppe reserviert ist.

Während Ufer- und Reticulumzellen von einer Reihe von Autoren für identisch erklärt werden (KIYONO (1914), ALFEJEW (1924), MAXIMOW (1927)), hält MARCHAND (1913) sie für verschiedene Zellen, weil entschieden sei, daß die Reticulumzellen verästelt seien und in den Knotenpunkten der Gitterfasernetze säßen, während die Uferzellen platte Zellen seien und die groben Balken der Sinus auskleiden. ASCHOFF (1926) meint, daß sie zwar zusammengehörten, aber nicht völlig eins seien, da die Reticulumzellen langsamer und schwächer speichern als die Uferzellen.

Was die Form der Uferzellen betrifft, so kann man sich leicht davon überzeugen, daß auch sie vielfach verästelte Zellen sind. Wie MAXIMOW (1927) besonders hervorgehoben hat, lassen sich ihr Cytoplasma und ihre Fasern „ohne jede Grenze unmittelbar in die entsprechenden Bestandteile des allgemeinen Reticulums verfolgen“. Sie unterscheiden sich morphologisch nur durch ihre äußeren, den lokalen mechanischen Bedingungen angepaßten Formen. Auch histogenetisch sind sie nach ALFEJEW (1924) einheitlicher Natur.

Die Angabe ASCHOFF's (1926), daß die Reticulumzellen im Gegensatz zu den Uferzellen nur wenig speichern und phagocytieren, scheint mir nicht unbedingt Ausdruck einer verschiedenen Funktion dieser Zellen, sondern eher durch die Farbstoffverteilung und durch die Strömungsverhältnisse in den Lymphknoten

bedingt zu sein, auf deren Wichtigkeit kürzlich RADT (1929) wieder hingewiesen hat. Bei Erkrankungen und Experimenten, die im lymphatischen Gewebe Kernzerfall hervorvorrufen, habe ich in Übereinstimmung mit vielen anderen Autoren, auch in den Reticulumzellen immer eine hochgradige Phagocytose gesehen. Auch in anscheinend normalem lymphatischem Gewebe kann man das oft genug beobachten. Dieses zeigt, daß die Reticulum- und Uferzellen auch funktionell einheitlicher Natur sind und von ASCHOFF mit Recht als reticuloendothelialer Apparat im engeren Sinne zusammengefaßt sind.

Ob man in den Lymphsinus noch eigentliche Endothelien unterscheiden muß, die auch die Form der gewöhnlichen Endothelien haben und nach ORSOS (1926) und nach MALLORY und PARKER (1927) keine Gitterfasern bilden, oder ob diese Zellen vielleicht schon in Ablösung begriffene Reticuloendothelien sind, ist unentschieden. Sie sollen im weiteren nicht von den Reticuloendothelien unterschieden werden.

Die undifferenzierten mesenchymalen Elemente der lymphatischen Organe nenne ich mit MAXIMOW (1927) „undifferenzierte Mesenchymzellen". Sie sind im Gegensatz zu den Reticuloendothelien klein und unscheinbar (Taf. XI, Abb. 1). Sie haben einen länglichen Kern mit wechselndem Chromatingehalt und mit sehr kleinen dunklen Nukleolen und sehr wenig schwach gefärbtes Protoplasma, das oft überhaupt nicht zu erkennen ist. Sie speichern nur sehr wenig. Ob sich außer diesen Verschiedenheiten den Reticuloendothelien gegenüber auch Unterschiede in bezug auf die Gitterfaserbildung finden, ist nicht untersucht worden. Vielleicht sind nur die Reticuloendothelien Gitterfaserbildner, und ist die Eigenschaft, Fasern zu bilden, wie die der Phagocytose, bereits Ausdruck einer bestimmten Differenzierung. Wenn die undifferenzierten Mesenchymzellen überhaupt Gitterfasern bilden, tun sie es wohl nur sehr wenig. In diesem Sinne sprechen z. B. die schönen Abbildungen von HEIDENHAIN (1911) und ORSOS (1926), deren Gitterfaserbildner alle den Reticuloendothelien und nicht den undifferenzierten Mesenchymzellen entsprechen.

Während der reticuloendotheliale Apparat der lymphatischen Organe in den letzten Jahrzehnten vielfach untersucht worden ist, ist das eigentliche lymphatische Gewebe sehr vernachlässigt worden. Erst durch HELLMAN's groß angelegte Untersuchungen (1913/14, 1918/19, 1921, 1926) hat es besonders in der Pathologie erneutes Interesse gefunden. Seine Untersuchungen wie die seiner Nachfolger beschäftigen sich hauptsächlich mit der Bedeutung der sog. Keimzentren.

Über die Funktion des lymphatischen Gewebes wissen wir, außer daß es Lymphocyten produziert, sehr wenig. Wir wissen nicht, ob die parenterale Verdauung ausschließlich im reticuloendothelialen Apparat stattfindet, oder ob nicht z. B. bestimmte Eiweißkörper von den Lymphocyten verdaut werden, wie HOFMEISTER (1885, 1886, 1887),

ASCHOFF (1924) und sein Schüler UCHINO (1925) vermutet haben. Auch morphologische Fragen, wie die der Entstehung und der prospektiven Potenzen der Lymphocyten, wie die der Beziehung der Lymphocyten zu dem reticuloendothelialen Apparat, ja selbst Fragen nach den Beziehungen der Sekundärknötchen zueinander und zum lymphoiden Gewebe waren so gut wie ungeklärt.

Meine Untersuchungen, welche ich 1925 im ASCHOFF'schen Institut begonnen und dann im ROCKEFELLER Institut fortgesetzt habe, betreffen besonders das eigentliche lymphatische Gewebe und zwar die oben angedeuteten morphologischen wie funktionellen Fragen. Dabei habe ich zuerst mit morphologischen und experimentellen Methoden die Histologie dieses Gewebes neu bearbeitet. Teilergebnisse dieser Untersuchungen habe ich schon früher (1929 a–d) mitgeteilt. Nachdem die Histologie klargestellt war, habe ich Versuche über die Lymphopoëse und das Werden und Vergehen der Sekundärknötchen angestellt. Erst, wenn auch die im Gange befindlichen Versuche über die prospektiven Potenzen der Lymphocyten abgeschlossen sind, will ich mich genauer mit der Funktion des lymphatischen Gewebes und der Lymphocyten beschäftigen.

Bevor ich nun zur Beschreibung meiner Untersuchungen übergehe, muß ich noch einige allgemeine Bemerkungen über Material und Färbemethoden vorausschicken.

Allgemeines über Material und Färbemethoden

Um beim Studium lymphatischer Gewebe Irrtümer zu vermeiden, sollte man nur Material von gesunden Tieren benutzen. Krankheiten des Organismus gehen oft mit Hyperplasie, häufiger noch—und zwar in den meisten tödlich verlaufenden Fällen—mit Atrophie und mit starken Veränderungen der histologischen Struktur dieser Gewebe einher. Als Beispiel füge ich hier einige Lymphknotengewichtszahlen von 20 Kaninchen an, die sich nur dadurch unterschieden, daß sie teils infolge bestimmter experimenteller Behandlung gestorben waren und teils dieselbe überlebt hatten. Die rechten und linken Popliteallymphknoten der gestorbenen Tiere wogen durchschnittlich nur 0,12 und 0,05 g, während die der am Leben gebliebenen 0,27 und 0,1 g wogen. Das durchschnittliche Normalgewicht dieser Lymphknoten war 0,1 g.

Als weitere Quelle für Irrtümer kommen postmortale Veränderungen in Frage. Um mir über ihre Bedeutung ein Bild zu machen, habe ich Stückchen von peripheren und mesenterialen Lymphknoten, von Milz und Blinddarm zweier Kaninchen, bei denen ich auch in den peripheren Lymphknoten FLEMMING'sche Sekundärknötchen erzeugt hatte, teils frisch, teils verschieden lange nach dem Tode

fixiert, indem die letzteren teils auf Eis und teils bei Zimmertemperatur aufgehoben wurden. Hierbei zeigte sich, daß schon 2 Stunden nach dem Tode sowohl bei Eis wie Zimmertemperatur postmortale Veränderungen deutlich waren, wenn auch nur geringfügiger Art. 18 Stunden nach dem Tode waren diese Veränderungen bei Aufbewahrung auf Eis weiter fortgeschritten und bei Zimmertemperatur schon hochgradig.

Die postmortalen Veränderungen sind sehr einheitlicher Natur. Das Gewebe erscheint mehr und mehr aufgelockert. Die Zellen liegen weiter voneinander entfernt und verkleinern sich mehr und mehr. Die Zahl der Mitosen nimmt ab. Die Kerne verklumpen und werden unscharf und den tingiblen Körperchen immer ähnlicher. Aber auch noch 18 Stunden nach dem Tode sieht man selbst bei Aufbewahrung in Zimmertemperatur noch einige wohlgeformte große Lymphocyten und vereinzelte Kernteilungsfiguren.

Bei meinen Untersuchungen wurden meist nur getötete Tiere benutzt und auch kranke Tiere ausgeschlossen. Nur im 4. Experiment des 2. Abschnitts kamen der Natur des Experimentes entsprechend auch gestorbene Tiere zur Untersuchung.

Zur Färbung von Gewebsschnitten habe ich die verschiedensten Methoden angewandt. Als Routinefärbungen benutzte ich zunächst Eisenhämatoxylin-Eosin und die Methylenblau-Eosinfärbung nach MALLORY (MALLORY und WRIGHT (1924)). Später benutzte ich nur noch die letzte. Sie steht nach meinen Erfahrungen der Azur II-Eosinfärbung von MAXIMOW (1909) in nichts nach, sondern hat eher einige technische Vorzüge. Zur Darstellung der Gitterfasern benutzte ich die sog. Azanfärbung, d. h. die von HEIDENHAIN (1915) vorgeschlagene Modifikation der Bindegewebsfärbung von MALLORY, und die Versilberungsmethode von BIELSCHOWSKY-MARESCH. Besonders schöne Resultate erzielte ich mit der Modifikation der letzteren durch LAIDLAW (1929). Als Plasmazellenfärbung benutzte ich zunächst die Methylgrün-Pyroninfärbung und später nur noch die von HITCHCOCK und mir (1930) angegebene Methode mit Malachitgrün und Akridinrot, da diese auch bei ZENKER-Fixation, welche zur Methylenblau-Eosinfärbung nötig ist, gute Resulte gibt. Außerdem lassen sich viel mehr Einzelheiten in der Zellstruktur und Abstufungen in dem Rot des Protoplasmas erkennen.

ABSCHNITT I

Die Histologie des Lymphatischen Gewebes

Wenn man von lymphatischem Gewebe spricht, so sollte man nach ASCHOFF (1926) darunter nur ein Gewebe verstehen, welches durch den Besitz von Sekundärknötchen ausgezeichnet ist. Lymphoides Gewebe ist ein mehr diffuses Gewebe, das sich, wie ich schon früher ausgeführt habe (1929 a), durch seinen Bau von den echten Sekundärknötchen unterscheidet. Es ist nur als ein Grundgewebe aufzufassen, in

welchem Sekundärknötchen entstehen und vergehen und welches z. T. von ihnen neugebildet wird. Diese ASCHOFF'sche Einteilung ist in der letzten Zeit vielfach angegriffen worden. So hat NORDMANN (1928) hervorgehoben, daß man in den Lymphknoten Mark und Rinde nicht streng unterscheiden könne. Darauf kommt es aber gar nicht an. ASCHOFF wollte nur das geformte, Sekundärknötchen enthaltende Gewebe von dem sie umgebenden, ungeformten, diffusen Grundgewebe unterscheiden. Wie wichtig seine Einteilung für das Verständnis des lymphatischen Gewebes ist, wird in dieser Arbeit wiederholt gezeigt werden.

Bei der Untersuchung von lymphatischem Gewebe und besonders bei der Beurteilung von Experimenten ist nun die regionäre Bedeutung von großer Wichtigkeit. Sie ist einerseits durch die Verteilung des lymphatischen Gewebes in den verschiedenen Säfteströmen des Körpers und andererseits durch die regionäre Anordnung zu den verschiedenen Organen bedingt. Man kann nicht erwarten, wie es immer noch geschieht, bei Peritonitis in dem lymphatischen Gewebe der Milz die wesentlichen Veränderungen zu finden, sondern muß sie in den zur Peritonealhöhle regionären Lymphknoten suchen.

Nach ASCHOFF (1926) müssen wir beim lymphatischen Gewebe nach Einschaltung in die Säfteströme des Körpers 3 Gruppen unterscheiden:

1. Das lymphatische Gewebe der Lymphknoten. Es besitzt sowohl zuwie abführende Lymphgefäße und ist in den eigentlichen Lymphstrom eingeschaltet.

2. Das lymphatische Gewebe in den Schleimhäuten. Es hat nur abführende Lymphgefäße und ist in den Saftstrom inseriert, der von den Schleimhäuten ins Innere des Organismus führt.

3. Das lymphatische Gewebe in der Milz. Es hat weder ab- noch zuführende Lymphgefäße und ist in die Blutbahn eingeschaltet.

Die beiden ersten Gruppen zerfallen durch ihre Abhängigkeit von verschiedenen Organen wieder in Untergruppen, worauf besonders NORDMANN (1928), GOSSMANN (1929) und FORKNER (1929) hingewiesen haben.

Das lymphatische Gewebe ist in den verschiedenen Organen sehr einheitlich gebaut. Prinzipielle Unterschiede hat man trotz extra darauf gerichtete Untersuchungen nicht finden können. In der Milz und in den Schleimhäuten finden sich zwar einige Besonderheiten. Ich werde im folgenden nur die Lymphknoten genauer besprechen, da sie die eigentlichen lymphatischen Organe sind und am übersichtlichsten gebaut erscheinen. Anschließend werde ich auf die erwähnten

Besonderheiten in Milz und Schleimhäuten hinweisen. Da wir über das Stützgewebe des lymphatischen Gewebes schon ausgezeichnete Arbeiten besitzen (RÖSSLE und YOSHIDA (1909), FERGUSON (1911), HEUDORFER (1921) und ORSOS (1925 a, 1926)), werde ich es hier nur soweit berücksichtigen, als zum Verständnis des Parenchyms nötig erscheint.

A. Das lymphatische Gewebe in den Lymphknoten

Wenn auch die Lymphknoten verschiedener Menschen und Tiere, ja selbst die einer einzelnen Lymphknotengruppe bei einem Individuum ganz verschiedene Bilder darbieten können, so liegt ihnen doch mit wenigen Ausnahmen ein gemeinsamer Bauplan zugrunde. Seit den

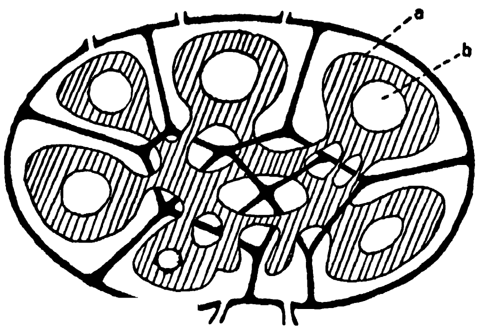

ABB. 1. Schema eines Lymphknotens nach STÖHR-MÖLLENDORFF (1922). Die Rinde besteht aus einzelnen durch Septen abgeteilte Follikel (a), in deren Mitte die sog. Keimzentren liegen (b).

60 er Jahren des vorigen Jahrhunderts finden wir ein Lymphknotenschema in der Literatur (Abb. 1), daß sich in den Lehrbüchern und Arbeiten bis auf den heutigen Tag erhalten hat (STÖHR-MÖLLENDORFF (1922), NORDMANN (1928)). Nach Ansicht dieser Autoren wurde die Rinde durch Septen, welche von der bindegewebigen Kapsel ausgingen, in einzelne „Rindenknoten" oder „Follikel" (a) geteilt, in deren Mitte die später von FLEMMING (1885) genauer beschriebenen „Keimzentren" lagen (b).

Erst durch HEUDORFER (1921) wurde ein neues Lymphknotenschema eingeführt (Abb. 2), welches vor allem die Verhältnisse, welche Sinus, Markstränge und Blutgefäße betreffen, richtig stellte. Was das

lymphatische Gewebe betrifft, zeigte er zum erstenmal, daß die Rinde nicht aus einzelnen durch Septen abgeteilten „Follikeln“ besteht, sondern daß sie das Mark „mantelartig umfaßt“ und nur von einzelnen Trabekeln samt begleitenden Sinus in radiärer Richtung durchzogen wird. Nach seinen Untersuchungen, welche wie die der früheren Autoren fast ausschließlich an mesenterialen Lymphknoten vorgenommen wurden, liegen mitten in der Rinde, in der Regel in mehrfacher Schichtung, wie auch vereinzelt in den Marksträngen die „Lymphfollikel“, welche nach den Abbildungen und Beschreibungen des Verfassers mit FLEMMING's Sekundärknötchen identisch sind. Wenn auch zugegeben werden muß, daß solche Bilder

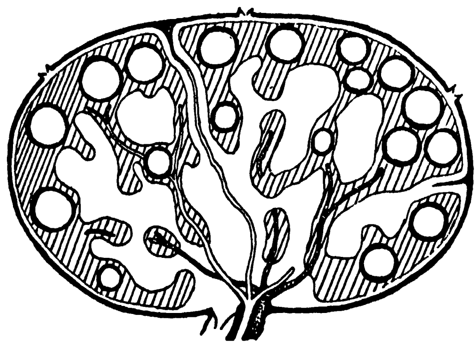

ABB. 2. Schema eines Lymphknotens nach HEUDORFER (1921). Die Rinde umfaßt das Mark „mantelartig“ und wird von einzelnen Trabekeln in radiärer Richtung durchzogen. In der Mitte der Rinde und in den Marksträngen liegen die „Lymphfollikel“ oder sog. Keimzentren.

gelegentlich vorkommen, so entsprechen sie jedoch nicht dem normalen Bauplan eines Lymphknotens. Sie sind vielmehr, wie wir später sehen werden, Ausdruck eines bestimmten Reizzustandes (vgl. Abb. 32 u. 35).

Der Bauplan eines normalen Lymphknotens, wie er sich aus meinen Untersuchungen (1929 a–c) ergeben hat, ist in Abb. 3 wiedergegeben. Die Rinde bedeckt das Mark in der von HEUDORFER angegebenen Weise. In der Rinde liegen die verschiedenen Sekundärknötchen. Die echten Sekundärknötchen oder die Lymphfollikel HEUDORFER's (b–d) liegen aber nicht, wie HEUDORFER beschrieben hat, in mehrfacher Schicht mitten in der Rinde und in den Marksträngen, sondern

in ungereizten Lymphknoten nur in einfacher Schicht dicht unter den Randsinus, und zwar in der äußersten Schicht der Rinde (siehe Abb. 4, 6, 8 u. 13). Außer den echten Sekundärknötchen enthält die Rinde sehr große von mir als Pseudosekundärknötchen beschriebene Gebilde (a), die sich von den echten Sekundärknötchen durch ihren Bau und ihre Lage grundsätzlich unterscheiden. Während Pseudosekundärknötchen nichts anderes als lymphoides Gewebe mit allen Eigenschaften dieses Gewebes darstellen, sind die echten Sekundärknötchen durch besondere Merkmale gekennzeichnet, die sie scharf von dem lymphoiden Gewebe unterscheiden. Bevor ich hierauf genauer eingehen kann, muß ich noch einige Worte über die Nomenklatur anfügen.

Schon FLEMMING (1885) beschwerte sich darüber, daß der Name Sekundärknötchen einmal „für ‚Rindenknoten' und den ähnlichen, hier und da auch im Innern auftetenden größeren Ballen" und ein andermal für Dinge gebraucht würde, die „mit (seinen) Sekundärknötchen, keineswegs aber mit den ganzen Rindenknoten zu vergleichen" seien. Dabei rechnete er seinen Sekundärknötchen „die dunkle Schale" zu, wie aus seiner Beschreibung (1885, S. 61 u. 62) und aus seinen Abbildungen mit Deutlichkeit hervorgeht, wie ich auch schon früher (1929a) im Gegensatz zu neueren Autoren betont habe. Als dann HEUDORFER (1921) gezeigt hatte, daß man die Rinde nicht in „Rindenknoten" oder „Follikel" aufteilen könne, sondern diese ein kontinuierlicher „Mantel" sei, und als von GROLL und KRAMPF (1920/21) die soliden Sekundärknötchen eingeführt wurden, kam es zu einer noch größeren Verwirrung in der Literatur. In vielen Arbeiten kann man der Beschreibung überhaupt nicht mehr entnehmen, welche Knötchen gemeint sind. Im allgemeinen hat man wohl die Vorstellung, daß die Rinde Primärknötchen (Follikel, Rindenknoten) enthält, in deren Mitte Sekundärknötchen entstehen und vergehen. Klare Angaben über die von mir beschriebenen Pseudosekundärknötchen habe ich überhaupt nicht gefunden. Auch was MAXIMOW (1927) als Primärknötchen beschrieben hat, entspricht nicht den Pseudosekundärknötchen, da er angibt, das „Keimzentrum" läge meist in Einzahl in der Mitte des Primärknötchens. Diese Beschreibung wie seine Abb. 51 entsprechen einem typischen echten Sekundärknötchen mit breiter Randzone.

Da die physiologische Bedeutung der im lymphatischen Gewebe auftretenden Knötchen noch nicht hinreichend bekannt ist, müssen wir uns einstweilen mit einem beschreibendem Namen begnügen. Sie alle sind nun, wie ich im 2. Abschnitt dieser Arbeit zeigen werde, genetisch einheitlicher Natur. Man kann sie deshalb auch alle mit einem einheitlichen Namen belegen. Da nun FLEMMING (1885), der als erster diese Knötchen eingehender beschrieben hat, ihnen den

Namen Sekundärknötchen gegeben hat und sich dieser Name in der Literatur weitgehend eingebürgert hat, und er auch der Natur der Knötchen am besten nahe kommt, scheint es mir am besten, den Namen Sekundärknötchen einstweilen beizubehalten.

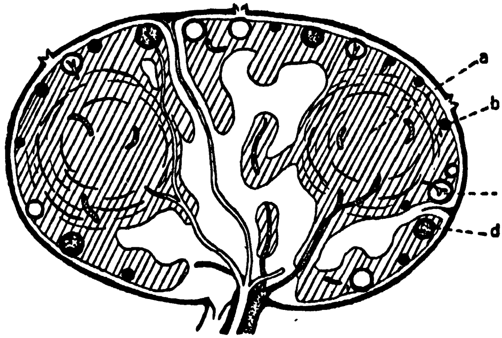

Abb. 3. Mein Lymphknotenschema. Die Rinde enthält knotenförmige Verdickungen, welche ich als Pseudosekundärknötchen bezeichnet habe (a). Die echten Sekundärknötchen liegen am äußeren Rande der Rinde. Man muß bei ihnen solide (b), Flemming'sche Sekundärknötchen oder die sog. Keimzentren (c) und Übergangssekundärknötchen (d) unterscheiden.

1. Die echten Sekundärknötchen

Die echten Sekundärknötchen sind mehr oder weniger kugelig oder eiförmig gebaut und durch ein retikuläres Netzwerk verschiedener Dichte von dem umgebenden lymphoiden Gewebe abgegrenzt. Das Netz ist, wie wir wissen, Ausdruck eines zentrifugalen Wachstumsdruckes. Je größer die Sekundärknötchen sind und je stärker die Gitterfasern vor der Entwicklung der Sekundärknötchen im Gewebe ausgebildet waren, um so dichter wird ihre retikuläre Begrenzung sein. Die echten Sekundärknötchen selber enthalten in ihrem Inneren im Gegensatz zum lymphoiden Gewebe nur spärlich feine Gitterfasern als Ausdruck ihrer Jugend. Erst in Übergangssekundärknötchen, in der Auflösungsphase der Flemming'schen „Keimzentren", sehen wir auch hier reichlicher Gitterfasern auftreten.

Wie ich schon früher angegeben habe, sind die echten Sekundärknötchen nur von arteriellen Präkapillaren und Kapillaren versorgt, während sie niemals Venen enthalten. Die Knötchen sitzen auf den Präkapillaren wie Kirschen auf ihrem Stiel. Hueck (1927) und sein

Schüler JÄGER (1929) haben die Blutversorgung der Sekundärknötchen der Milz genauer studiert. Nach ihrer Beschreibung zieht die Arteriole in gerader Richtung bis etwa zur Mitte des Sekundärknötchens. Hier entspringt dann eine größere Zahl dünner Kapillaren, die sich gleichmäßig nach allen Richtungen verteilen. Meine Beobachtungen bestätigen, von einigen Variationen abgesehen, diesen Befund auch in den Lymphknoten.

Auf Grund ihrer cellulären Zusammensetzung und ihrer Bedeutung müssen wir drei Arten von echten Sekundärknötchen unterscheiden.

ABB. 4. Kan. 320. Lymphknoten. Methylenblau-Eosin. 75 fache Vergr. Solide Sekundärknötchen am äußeren Rande der Rinde.

a) Solide Sekundärknötchen (Abb. 4). Sie sind zuerst von GROLL und KRAMPF (1920/21) eingehender beschrieben worden. Sie sind die kleinsten und genetisch jüngsten Sekundärknötchen. Ihr Durchmesser übertrifft nur selten 0,3 mm. Sie bestehen im stationären Zustand fast ausschließlich aus kleinen wohlgeformten Lymphocyten, die in geeigneten Schnitten deutlich konzentrisch um die Arteriole gelagert sind (1929 a, Abb. 4). Manchmal enthalten sie vereinzelte große und mittelgroße Lymphocyten, die im wachsenden Zustand dieser Knötchen vermehrt sind. Für gewöhnlich enthalten sie auch einige gleichmäßig verteilte Reticulumzellen und undifferenzierte

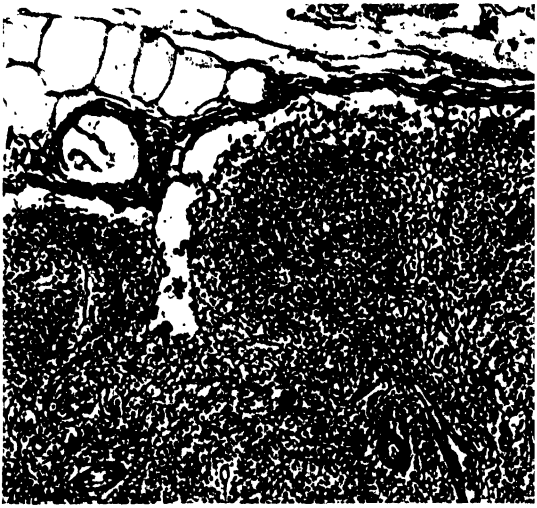

ABB. 5. Kan. 125. Lymphknoten. Silberimprägnation. 170 fache Vergr. Solides Sekundärknötchen am Rande der Rinde. Es ist arm an Gitterfasern und hat die des lymphoiden Gewebes deutlich verdrängt.

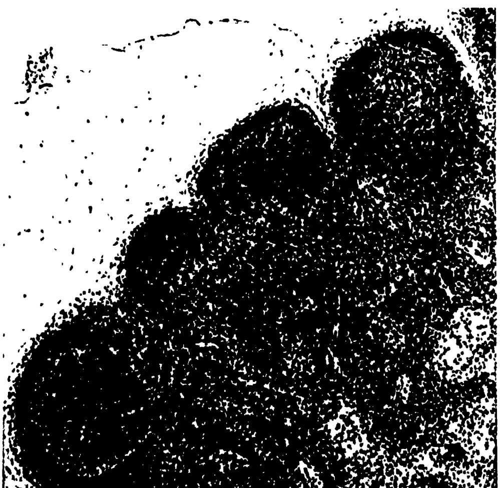

ABB. 6. Kan. 588. Lymphknoten. Methylenblau-Eosin. 70 fache Vergr. FLEMMING'sche Sekundärknötchen oder sog. Keimzentren am äußeren Rande der Rinde. Sie besitzen gut begrenzte helle Zentren und verschieden große Kappen.

Mesenchymzellen. Die soliden Sekundärknötchen sind sehr arm an Gitterfasern (Abb. 5). Ihre retikuläre Begrenzung ist meist deutlich.

b) FLEMMING's Sekundärknötchen oder die sog. „Keimzentren" (Abb. 6). Sie sind identisch mit HEUDORFER's (1921) Lymphfollikel. Sie treten erst im postfetalen Leben auf und sind im allgemeinen wesentlich größer als die soliden Sekundärknötchen und erreichen nicht selten einen Durchmesser von 0,75 mm. Sie bestehen meist aus einem hellen Kern und einer dunklen Schale oder der sog. Randzone. Die Randzone umgibt den hellen Kern mehr oder weniger gleichmäßig. Häufig, besonders im lymphatischen Gewebe der Schleimhäute, sitzt sie dem Kern wie eine Kappe auf. Gelegentlich, besonders in pathologischen Fällen, kann sie auch ganz fehlen.

Die Randzone der FLEMMING'schen Sekundärknötchen ist besonders von RÖHLICH (1928) und SCHWANEN (1929 a) eingehend untersucht worden. Während RÖHLICH versucht hat, die Form der Randzone allein durch innerhalb des Gitterfasersystems entstehende Spannungen und Zellverschiebungen zu erklären, die durch den Wachstumsdruck der Sekundärknötchen ausgelöst werden, führte SCHWANEN die Kappenbildung auf einseitig vom Epithel oder den Sinus ausgehende starke Lymphocyten bildende Reize zurück, wobei er sich auf die Lage der Kappe stützte, die meist dem Epithel oder den Sinus zugekehrt ist. Diese Erklärungen sind aber keineswegs ausreichend. Wie ich im 2. Abschnitt ausführlicher darstellen werde, hängt die Form der Randzone besonders von der örtlichen Entstehung der hellen Zentren und ihrer Beziehung von den Blutgefäßen ab, wobei dann mechanische Verhältnisse im Sinne RÖHLICH's von sekundärer Bedeutung sind.

Während sich die Randzone in ihrem Bau von dem der soliden Sekundärknötchen in nichts unterscheidet, ist das helle Zentrum ganz anders gebaut. Im stationären Zustand besteht es fast ausschließlich aus mittelgroßen Lymphocyten, worauf ich schon früher (1929 a) hingewiesen habe. Hierauf hat auch MAXIMOW (1927) besonderen Nachdruck gelegt. Das Protoplasma dieser mittelgroßen Lymphocyten ist im Gegensatz zu dem anderer Lymphocyten nur schwach basophil—auch hierin stimmt meine frühere Beschreibung mit der von MAXIMOW überein—und zeigt oft am Rande kleine knospenförmige Abschnürungen, wie sie schon von EHRLICH beobachtet worden sind (DOWNEY und WEIDENREICH (1912), CUNNINGHAM, SABIN und DOAN (1925), MAXIMOW (1927)). Meistens finden sich, besonders den Gefäßen entlang, einzelne große Lymphocyten, die

im wachsenden Zustand der Zentren vermehrt sind. Gelegentlich fehlen sie ganz, wie auch von MAXIMOW (1927) beobachtet. Kleine Lymphocyten sind für gewöhnlich auch nur in sehr spärlicher Zahl vorhanden. Ihre Kerne zeigen häufig als Ausdruck regressiver Umwandlung Hyperchromatose, Pyknose oder Karyorrhexis. Daß die kleinen Lymphocyten in den hellen Zentren nicht so wohlgeformt sind als die des übrigen lymphatischen Gewebes, ist auch MAXIMOW aufgefallen, wenn er dieser Tatsache auch eine andere Bedeutung

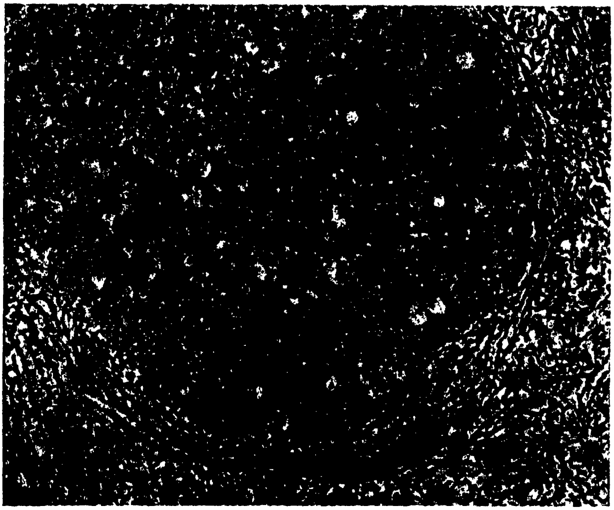

ABB. 7. Kan. 139. Lymphknoten. Silberimprägnation. 170 fache Vergr. FLEMMING'sches Sekundärknötchen. Es ist von einem starken Gitterfasernetz begrenzt, während sein Inneres frei von Gitterfasern ist.

zumißt als ich es tue (siehe später). Von den kleinen Lymphocyten zu den tingiblen Körperchen finden sich alle Übergänge. Letztere sind, wie wir seit den Untersuchungen von HEINECKE (1905) wissen, Lymphocytenkerntrümmer. Sie werden von den Reticulumzellen phagocytiert, in welchen man, besonders unter pathologischen Bedingungen oft große Mengen finden kann.

Die hellen Zentren enthalten, wie die soliden Sekundärknötchen, Reticulumzellen und undifferenzierte Mesenchymzellen. Wie auch MAXIMOW (1927) angegeben hat, sind sie meist leicht von den Lymphocyten zu unterscheiden. Trotz ihrer manchmal vermehrten Chroma-

tinmenge ist der Kern meist blaß und enthält für gewöhnlich nur kleine Nukleolen. Ihr Protoplasma ist im Gegensatz zu dem der Lymphocyten nur sehr schwach basophil oder azidophil.

Was die hellen Zentren der FLEMMING'schen Sekundärknötchen vor allem auszeichnet, ist ihr stets mehr oder weniger reichlicher Gehalt an Kernteilungsfiguren. Während FLEMMING (1885) annahm, daß sie vorwiegend den Lymphocyten angehören, glaubte BAUMGARTEN (1885), daß sie in den retikulären Zellen lägen. Aus seiner Beschreibung ist aber nicht zu entnehmen, ob er damit die Reticulumzellen oder die undifferenzierten Mesenchymzellen gemeint hat, ja nicht einmal, ob er nicht die großen und mittelgroßen Lymphocyten mit seinen retikulären Zellen identifiziert hat. RIBBERT (1890) drückte sich präziser aus und glaubte die Kernteilungsfiguren den „Reticuloendothelien, die durch größere helle, ovale oder runde Kerne" ausgezeichnet waren, zuschreiben zu müssen und daß die „typischen Lymphzellen" niemals Kernteilungsfiguren enthielten. Wie wir heute wissen, gehören die Kernteilungsfiguren besonders den großen und mittelgroßen Lymphocyten an, während die fixen Elemente je nach Beanspruchung wechselnde Mengen enthalten. Dabei möchte ich aber im Gegensatz zu MAXIMOW (1927) hervorheben, daß die großen Lymphocyten und ihre Vorstufen, und nicht die mittelgroßen Lymphocyten besonders reich an Kernteilungsfiguren sind. Ich habe mich davon immer wieder überzeugen können. Auch sind die Kernteilungsfiguren wie die großen Lymphocyten besonders entlang den Gefäßen angeordnet, wie auch WEST (1924) beobachtet hat.

So lange die hellen Zentren noch klein sind, ist ihre Abgrenzung gegen die Randzone meist unscharf (siehe auch MAXIMOW (1927) Abb. 61). Je größer sie werden, um so schärfer wird diese Grenze. Außen um die Randzone oder in ihr findet sich das für alle Sekundärknötchen charakteristische Gitterfasernetz (Abb. 7), das bei fehlender Randzone mit der Grenze des hellen Zentrums zusammenfällt. Im Inneren dieser Sekundärknötchen finden sich nur spärlich unscheinbare Gitterfasern, oder diese fehlen auch ganz (Abb. 7).

c) Übergangssekundärknötchen (Abb. 8). Sie sind mit den Auflösungsstadien von HEIBERG (1923) und mit der Ruhephase MAXIMOW's (1927) identisch, nicht aber mit den ruhenden Sekundärknötchen von GROLL und KRAMPF (1920/21), die wahrscheinlich den soliden Sekun-

därknötchen entsprechen. Wenn ich die Übergangssekundärknötchen als besondere von den übrigen Sekundärknötchen abgetrennt habe, finde ich mich damit in Übereinstimmung mit MAXIMOW (1927). Sie sind sowohl histologisch anders gebaut als auch funktionell von ganz anderer Bedeutung. Von ihnen finden sich alle Übergänge zu pathologischen Formen, wie zu epitheloiden, hyalinen und amyloiden Sekundärknötchen. Ob man von ihnen morphologisch als pathologische Form eine retikuläre Gruppe unterscheiden kann,

ABB. 8. Kan. 481. Lymphknoten. Methylenblau-Eosin. 70 fache Vergr. Übergangssekundärknötchen, d. h. FLEMMING'sche Sekundärknötchen, deren helle Zentren in Auflösung begriffen und nicht mehr deutlich von der Randzone abgegrenzt sind.

wie ROTTER (1927) es tut, ist fraglich, da, wie wir sehen werden, die Übergangssekundärknötchen gerade durch ihren Reichtum an Reticulumzellen charakterisiert sind. Immerhin gibt es Fälle, wo man sie mit Sicherheit als pathologisch bezeichnen muß. Ich habe solche Sekundärknötchen bei einem 34 cm langem Fetus beschrieben und abgebildet (1929 b), dessen Mutter an Pneumonie erkrankt war. In diesem Falle waren sie anscheinend aus soliden Sekundärknötchen hervorgegangen. Für gewöhnlich treten die Über-

gangssekundärknötchen erst im postfetalen Leben auf, wenn sich bereits FLEMMING'sche Sekundärknötchen gebildet haben.

Die Größe der Übergangssekundärknötchen stimmt meist mit der der FLEMMING'schen überein oder übertrifft sie. An Häufigkeit übertreffen sie meist alle anderen Sekundärknötchen (siehe auch SCHUMACHER (1897) und HEIBERG (1923)). Sie bestehen, wie FLEMMING'S Sekundärknötchen, aus hellem Zentrum und dunkler Randzone. Die Abgrenzung der Zentren gegen die Randzone ist meist unscharf und

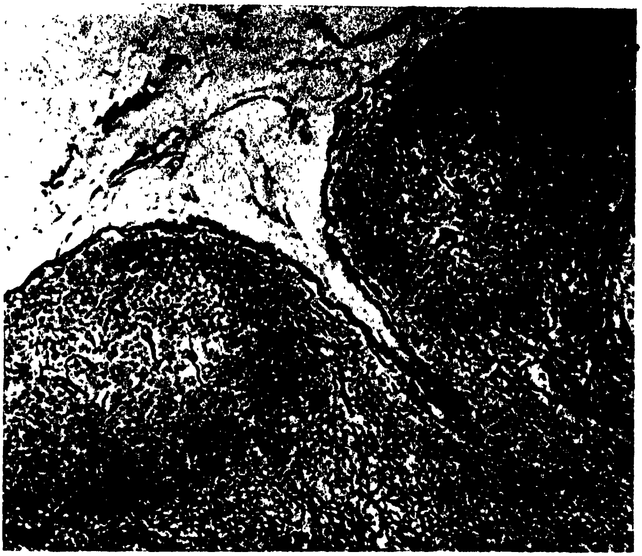

ABB. 9. Kan. 125. Lymphknoten. Silberimprägnation. 170 fache Vergr. Übergangssekundärknötchen mit reichlich Gitterfasern.

kann schließlich ganz verloren gehen. Die retikuläre Abgrenzung gegen das lymphoide Gewebe wechselt je nach ihrer Größe.

Das helle Zentrum ist im Gegensatz zu dem der FLEMMING'schen Sekundärknötchen sehr ungleichmäßig gebaut und viel zellärmer. Große Lymphocyten fehlen meist völlig. Wenn ein solches Zentrum aber wieder zu wachsen anfängt, treten auch wieder große Lymphocyten in ihm auf. Der Gehalt an mittleren Lymphocyten variiert sehr stark. Sie werden um so spärlicher, je weiter die Auflösung fortgeschritten ist. Damit einher geht eine zunehmende Kernverän-

derung im Sinne regressiver Umwandlung. Kleine Lymphocyten finden sich in zunehmender Zahl, doch sehen sie nicht so wohlgeformt aus wie die der soliden Sekundärknötchen. Auch bei ihnen findet man häufig regressive Kernveränderungen. Schließlich sieht man gelegentlich auch Plasmazellen.

Die Übergangssekundärknötchen sind besonders reich an retikulären Zellen, wie auch von MAXIMOW (1927) beschrieben und abgebildet (siehe seine Abb. 54). Ferner enthalten sie auch undifferenzierte

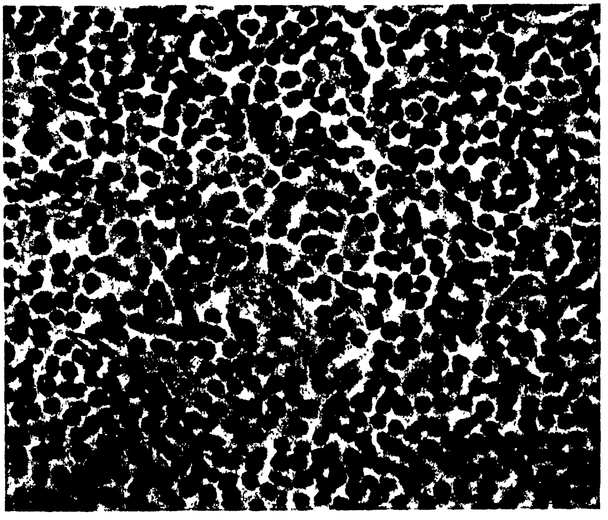

ABB. 10. Kan. 256. Lymphknoten. Methylenblau-Eosin. 580 fache Vergr. Lymphoides Gewebe, welches fast ausschließlich aus kleinen Lymphocyten besteht.

Mesenchymzellen. Die Reticulumzellen enthalten oft sehr reichlich Protoplasma, das vielfach zusammengeflossen erscheint. Je weiter die Auflösung fortgeschritten ist, um so mehr Gitterfasern treten in diesen Sekundärknötchen auf (Abb. 9), die beträchtliche Dicke erreichen können. Hiermit gehen regressive Veränderungen an den Gefäßen einher, wie auch von HUECK (1927) und JÄGER (1929) beobachtet.

Je weiter die Auflösung fortgeschritten ist, um so mehr ähneln die Übergangssekundärknötchen dem lymphoiden Gewebe oder den

Pseudosekundärknötchen. Wenn man sie nicht mehr von diesen unterscheiden kann, ist ihre Auflösung perfekt.

2. *Das lymphoide Gewebe*

Während die echten Sekundärknötchen definitiv geformte, wohl begrenzte Gebilde darstellen, ist das lymphoide Gewebe mehr diffus gebaut. Es dient den Sekundärknötchen gewissermaßen als Grundgewebe und füllt die Lücken zwischen ihnen aus. Es ist im Gegensatz zu den Sekundärknötchen reich an mehr oder weniger kräftig ent-

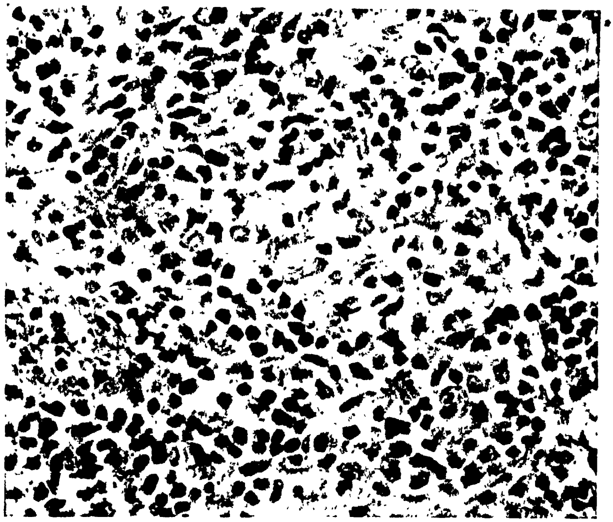

ABB. 11. Kan. 153. Lymphknoten. Methylenblau-Eosin. 580fache Vergr. Lymphoides Gewebe, welches reich an Reticulumzellen ist.

wickelten Gitterfasern, die ein dichtes Netzwerk bilden, in das die cellulären Elemente eingelagert sind. Im ruhenden Zustand enthält dieses Gewebe vorwiegend kleine Lymphocyten (Abb. 10), denen einige mittelgroße und vereinzelte große Lymphocyten beigemischt sein können. Im wachsenden Zustand treten, wie in den Sekundärknötchen, vermehrte große und mittlere Lymphocyten in ihnen auf. Außer echten Lymphocyten enthält das lymphoide Gewebe wechselnde Mengen Plasmazellen, deren Kern exzentrisch gelagert und deren Protoplasma durch eine Aufhellung neben dem Kern ausge-

zeichnet ist. Sie finden sich oft in großen Mengen in den Marksträngen.

Der Gehalt an Reticulumzellen wechselt stark. Gelegentlich, besonders unter pathologischen Bedingungen, können sie an Zahl über die Lymphocyten prädominieren (Abb. 11). Schließlich enthält dieses Gewebe auch mehr oder weniger reichlich undifferenzierte Mesenchymzellen, die besonders entlang den Gefäßen angehäuft sind.

Besonders charakteristisch für dieses Gewebe sind, wie ich früher beschrieben habe, eigenartig gebaute Venen, die in keinem anderen

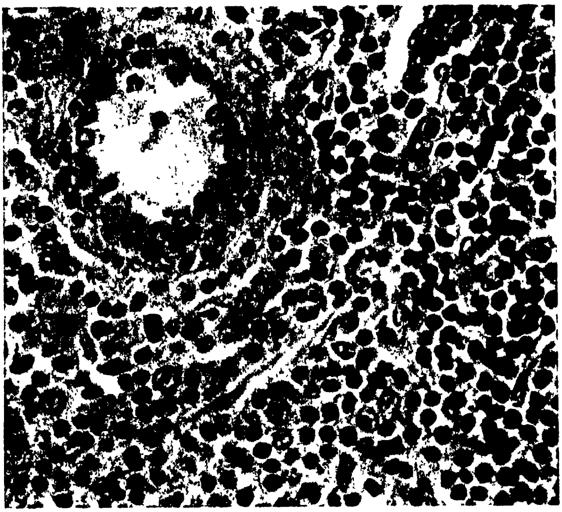

Abb. 12. Kan. 528. Lymphknoten. Methylenblau-Eosin. 575 fache Vergr. Vene im lymphoiden Gewebe mit epithelähnlichen Endothelien. Durch die Venenwand treten Lymphocyten in die Blutbahn über.

Gewebe vorzukommen scheinen. Sie sind zuerst von Schulze (1925) eingehender untersucht worden. Auch Maximow (1927) hat sie besonders erwähnt. Ihre Endothelien sind ungewöhnlich groß und zahlreich (Abb. 12). Sie haben große blasse Kerne und einen großen Protoplasmaleib, so daß sie fast wie Epithelien aussehen. Sie springen oft weit ins Lumen vor. Zwischen den Endothelien und in der basalen Schicht der Venen finden sich wechselnde Mengen besonders kleiner Lymphocyten, die hier, wie ich schon früher (1929 a und c) beschrieben habe, direkt ins Blut auswandern. Das Gleiche hatte

übrigens schon SCHUMACHER (1899) durch Vergleich von Arterien und Venen im Schnitt und durch Leukocytenzählungen in Arterien und Venen gezeigt.

3. *Die Pseudosekundärknötchen*

Die von mir (1929 a) beschriebenen Pseudosekundärknötchen stimmen darin mit den echten Sekundärknötchen überein, daß auch sie

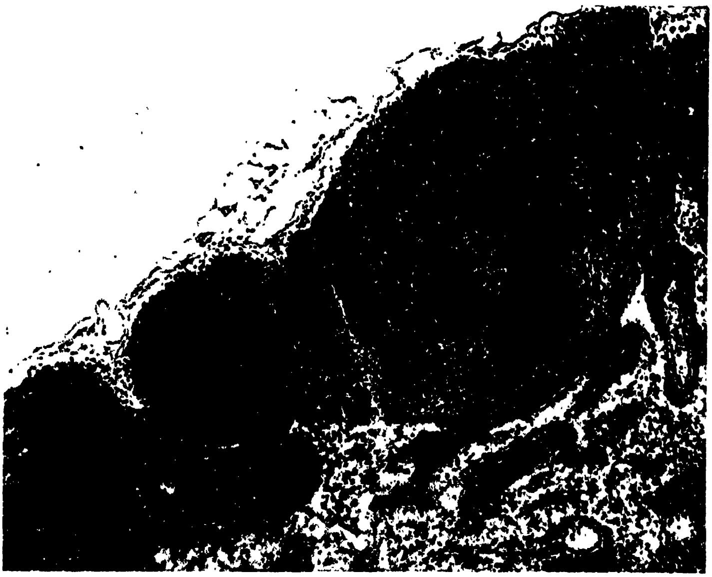

ABB. 13. Kan. 222. Lymphknoten. Methylenblau-Eosin. 75 fache Vergr. Die knötchenförmige Verdickung der Rinde ist ein Pseudosekundärknötchen, das wie lymphoides Gewebe zusammengesetzt ist. Am Rande der Rinde findet sich außerdem ein kleines solides Sekundärknötchen und ein FLEMMING'sches Sekundärknötchen oder Keimzentrum mit hellem Zentrum und Kappe.

wohl begrenzte, kugelige und eiförmige Gebilde sind (Abb. 13 und 14). Während die echten Sekundärknötchen aber stets am äußeren Rande der Rinde lokalisiert sind, nehmen die Pseudosekundärknötchen meist die ganze Breite der Rinde ein und bilden knötchenförmige Verdickungen derselben. Sie sind viel größer als die echten Sekundärknötchen. Ihr Durchmesser kann bis 3 mm und mehr betragen. Während die kleineren meist ein einheitliches Knötchen darstellen,

das unizentrisch gewachsen ist (Abb. 13), scheinen die größeren multizentrisch entstanden zu sein (Abb. 14), was besonders bei Gitterfaserfärbung deutlich ist (Abb. 15). Auf die Entstehung dieser Knötchen werden wir im zweiten Abschnitt zu sprechen kommen.

Die Pseudosekundärknötchen sind wie die echten Sekundärknötchen von einem Gitterfasernetz begrenzt, das bei großen Knötchen eine beträchtliche Dicke und Stärke erreicht (Abb. 15) und das der echten Sekundärknötchen weit übertrifft. Ihr retikuläres Innennetz wechselt.

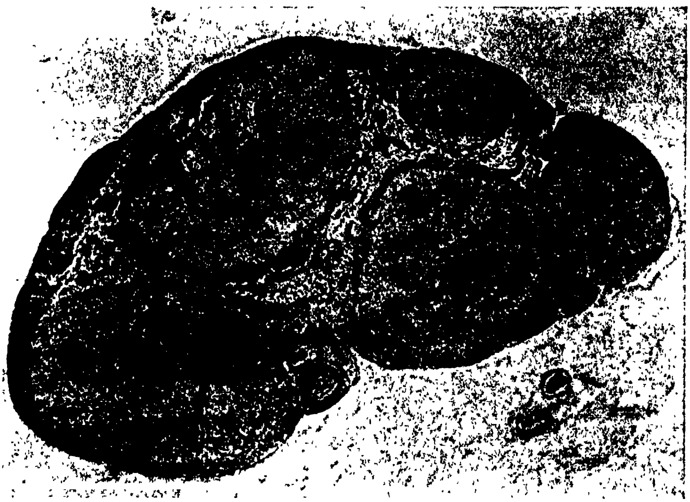

Abb. 14. Kan. 136. Längsschnitt durch einen Lymphknoten. Eisenhämatoxylin-Eosin. 42 fache Vergr. Die Rinde besteht aus 5 großen Pseudosekundärknötchen und enthält eine Anzahl echter Sekundärknötchen.

In jüngeren Pseudosekundärknötchen ist es nur schwach ausgebildet, während es in den älteren dem des lymphoiden Gewebes gleichkommt.

Die celluläre Zusammensetzung der Pseudosekundärknötchen unterscheidet sich in nichts von der des lymphoiden Gewebes. Auch sie sind vorwiegend aus kleinen Lymphocyten zusammengesetzt. Große und mittelgroße Lymphocyten fehlen im stationären Zustand meist völlig. Im wachsenden Zustand können sie aber reichlich große und mittelgroße Lymphocyten enthalten. Der Gehalt an Reticulumzellen wechselt wie im lymphoiden Gewebe. Undifferenzierte Mesenchymzellen finden sich besonders entlang den Blutgefäßen angehäuft.

Die Pseudosekundärknötchen enthalten schließlich, wie das lymphoide Gewebe, die für dieses Gewebe charakteristischen Venen, in welche wechselnde Mengen Lymphocyten einwandern. Die Venen sind hier oft besonders gut ausgebildet.

B. Das lymphatische Gewebe in den Schleimhäuten

Das lymphatische Gewebe der Schleimhäute liegt in der Tunica propria dicht unter dem Epithel. Es geht ohne scharfe Grenze in das

ABB. 15. Kan. 125. Lymphknoten. Silberimprägnation. 170 fache Vergr. Ausschnitt aus dem Rande eines Pseudosekundärknötchens mit starken, verdrängten Gitterfasern. Auch im Innern des Knötchens finden sich reichlich Gitterfasern.

umgebende Bindegewebe über. Es handelt sich auch hier um lymphoides Gewebe, in das je nach dem einzelne oder mehrere Sekundärknötchen eingelagert sind (Noduli lymphatici solitarii oder aggregati). Unter den echten Sekundärknötchen lassen sich auch hier solide, FLEMMING' sche und Übergangssekundärknötchen unterscheiden, die mit den echten Sekundärknötchen der Lymphknoten identisch sind und alle ihre Merkmale aufweisen. Auch das lymphoide Gewebe unterscheidet sich

in nichts von dem der Lymphknoten und ist auch hier durch die Art und Anordnung seines retikulären Netzes und der für das lymphoide Gewebe charakteristischen Venen (Abb. 16) ausgezeichnet. Gelegentlich habe ich auch Pseudosekundärknötchen gesehen, die wie lymphoides Gewebe zusammengesetzt sind und typische Venen enthalten.

Die FLEMMING'schen und Übergangssekundärknötchen der Darmschleimhaut und besonders des Blinddarms des Kaninchens sind oft

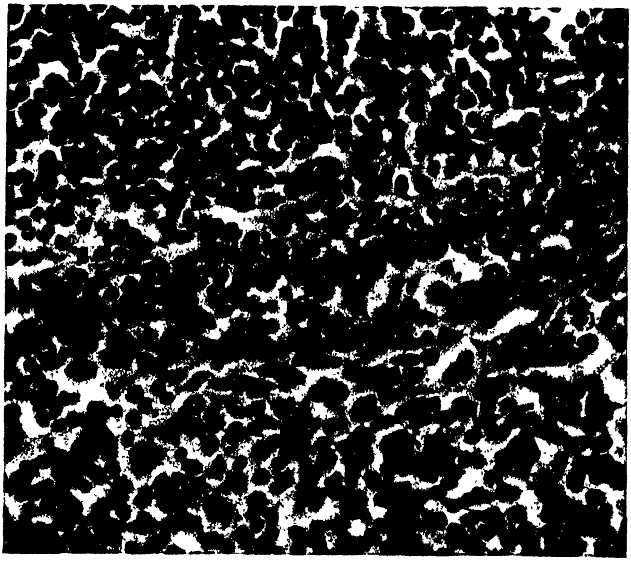

ABB. 16. Kan. 131. Tonsille. Eisenhämatoxylin-Eosin. 575 fache Vergr Vene im Längsschnitt.

auffallend reich an pigmentierten Substanzen (Abb. 17 und 18), welche nach LUBARSCH (1917) bei den Herbivoren regelmäßig in den Sekundärknötchen des Darmes gefunden werden. Man findet die gleichen Substanzen in großen Mengen in den Sinus der Mesenteriallymphknoten und in kleinen Mengen auch im lymphatischen Gewebe dieser Lymphknoten (Abb. 19) wie anderswo. Während SIMON (1909) dieses Pigment für ein hämatogenes gehalten hat, hat LUBARSCH (1917) es für Melanin erklärt. Wie man sich mit einer Eisenreaktion leicht überzeugen kann, enthält diese Substanz oft kleine eisenposi-

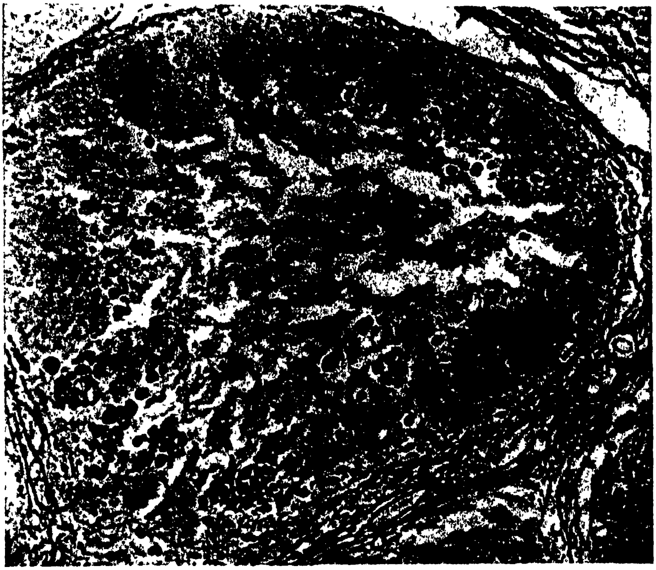

ABB. 17. Kan. 125. Blinddarm. Silberimprägnation. 170fache Vergr. Pigmenthaltige Zellen in einem FLEMMING'schen Sekundärknötchen.

ABB. 18. Kan. 11. Blinddarm eines 14 Tage alten Kaninchens. FISCHLER-Färbung. 60fache Vergr. FISCHLER-positives Pigment in den Sekundärknötchen.

tive Bestandteile. Als ganzes darf sie aber nicht als hämatogenes Pigment aufgefaßt werden, da sie eine starke FISCHLER-Reaktion gibt (Abb. 18 und 19) und sich mit Silbernitrat schwärzt (Abb. 17). Die positive FISCHLER-Reaktion spricht auch gegen die Annahme LUBARSCH's, daß es sich hier um Melanin handelt. Gegen seine Eigenschaft als eigentliches Abnutzungs-pigment spricht seine fast fehlende Färbbarkeit mit Fettfarbstoffen. Da unser Pigment außer den bisher erwähnten Reaktionen bei Zusatz von Schwefelsäure einen

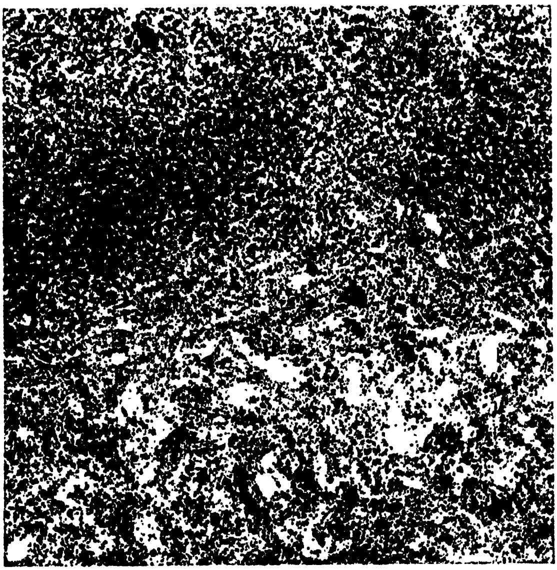

ABB. 19. Kan. 5. Mesenteriallymphknoten. FISCHLER-Färbung. 105 fache Vergr. Reichlich FISCHLER-positives Pigment in Rinde und Mark.

Farbumschlag in Blau bis Blaugrün gibt und sich auch im Bleichungsversuch wie ein Lipochrom verhält, müssen wir es als ein Lipochrom ansprechen.

C. Das lymphatische Gewebe in der Milz

In der Milz umscheidet das lymphatische Gewebe die mittleren und kleineren Arterien. Je nach Tierart sind die Arterien von einer dickeren oder dünneren, stets mehr oder weniger kontinuierlichen Lymphscheide umgeben (HELLSTEN (1928)), in welcher sie zentral

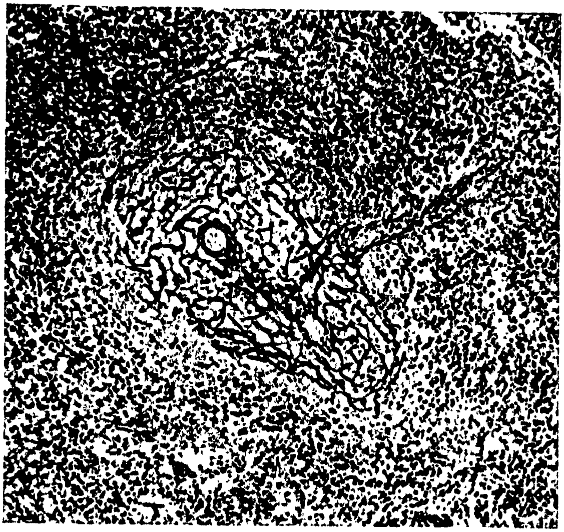

Abb. 20. Kan. 143. Milz. Silberimprägnation. 170 fache Vergr. Lymphscheide um eine Zentralarterie mit reichlich Gitterfasern.

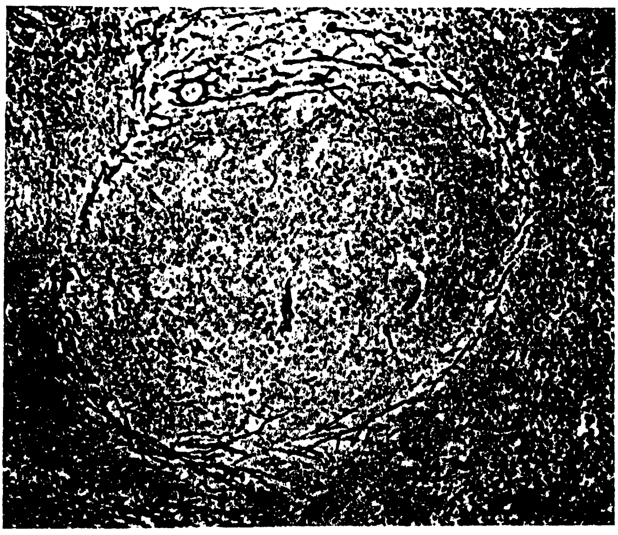

Abb. 21. Kan. 143. Milz. Silberimprägnation. 170 fache Vergr. Flemming'sches Sekundärknötchen in einer Lymphscheide außerhalb der Zentralarterie.

gelagert sind. Die Lymphscheiden sind nichts anderes als lymphoides Gewebe, wie durch ihre celluläre Zusammensetzung und besonders durch ihr retikuläres Netzwerk erwiesen ist (Abb. 20), in der an bestimmten Stellen echte Sekundärknötchen eingelagert sind. Unter den Sekundärknötchen können wir wieder solide, FLEMMING'sche (Abb. 21) und Übergangssekundärknötchen (Abb. 22) unterscheiden. Sie entwickeln sich stets neben den Zentralarterien (Abb. 21 und 22), wie das besonders von HUECK (1927) und seinen Schülern JÄGER (1929) und ONO (1930) studiert worden ist. Die Sekundärknötchen der

ABB. 22. Kan. 135. Milz. Silberimprägnation. 170 fache Vergr. Übergangssekundärknötchen mit reichlich Gitterfasern in einer Lymphscheide außerhalb der Zentralarterie.

Milz sind von einem besonders dichten Reticulumnetz abgegrenzt, was durch den großen Reichtum der Lymphscheiden an Gitterfasern erklärt ist. Pseudosekundärknötchen habe ich in der Milz nicht gesehen.

Die Lymphscheiden oder das lymphoide Gewebe der Milz unterscheiden sich nun dadurch von dem der anderen lymphatischen Organe, daß die für das lymphoide Gewebe charakteristischen Venen fehlen. Das ist nicht verwunderlich, wenn man bedenkt, daß die Venen Austrittsorte für Lymphocyten ins Blut darstellen. Da das lymph-

atische Gewebe der Milz schon direkt in die Blutbahn eingeschaltet ist und die Lymphscheiden immer nur verhältnismäßig dünn sind, können die hier gebildeten Lymphocyten direkt ins Blut einwandern und bedürfen keiner besonderen Venen, wie in den übrigen lymphatischen Organen.

ZUSAMMENFASSUNG DES ERSTEN ABSCHNITTS

Unter lymphatischem Gewebe verstehe ich mit ASCHOFF ein lymphoides Gewebe, in welches Sekundärknötchen eingelagert sind. Während das lymphoide Gewebe diffus angeordnet ist, sind die Sekundärknötchen kugelige oder eiförmige Gebilde, die je nach ihrer Größe durch ein mehr oder weniger deutliches Gitterfasernetz vom lymphoiden Gewebe abgegrenzt sind.

Unter den Sekundärknötchen muß man echte und Pseudosekundärknötchen unterscheiden. Die echten Sekundärknötchen sind im Gegensatz zum lymphoiden Gewebe arm an Gitterfasern und nur von arteriellen Präkapillaren und Kapillaren versorgt. Eigentliche Venen fehlen in ihnen. Auf Grund ihrer cellulären Zusammensetzung und Bedeutung müssen wir 3 Arten von echten Sekundärknötchen unterscheiden:

a) Solide Sekundärknötchen. Sie sind die kleinsten Sekundärknötchen und bestehen im stationären Zustand vorwiegend aus kleinen Lymphocyten.

b) FLEMMING'sche Sekundärknötchen oder die sog. Keimzentren. Sie haben ein helles Zentrum, welches vorwiegend aus mittelgroßen Lymphocyten besteht und reich an Kernteilungsfiguren ist, und eine dunkle, aus kleinen Lymphocyten zusammengesetzte Randzone oder Kappe, welche auch ganz fehlen kann. Bei wohl ausgebildeten Knötchen ist das helle Zentrum scharf gegen die Randzone abgesetzt. Übergänge von den mittelgroßen Lymphocyten des hellen Zentrums zu den kleinen Lymphocyten der Randzone fehlen für gewöhnlich.

c) Übergangssekundärknötchen. Sie sind Auflösungstadien der FLEMMING'schen Sekundärknötchen und zeigen alle Übergänge von diesen zum lymphoiden Gewebe.

Neben den echten Sekundärknötchen enthält das lymphatische Gewebe die von mir als Pseudosekundärknötchen bezeichneten Gebilde. Sie sind die größten Sekundärknötchen. Ihr Durchmesser kann 3

mm und mehr betragen, während der der echten Sekundarknötchen nur selten 0,75 mm übertrifft. Sie stimmen nur darin mit den echten Sekundärknötchen überein, daß auch sie wohlbegrenzte kugelige Gebilde sind. In ihrer cellulären Zusammensetzung unterscheiden sie sich aber in nichts vom lymphoiden Gewebe. Sie sind gewöhnlich reich an den für das lymphoide Gewebe charakteristischen Venen, die den Lymphocyten als Austrittsorte ins Blut dienen.

Das lymphatische Gewebe ist überall prinzipiell gleich gebaut. In den Lymphknoten bildet es die Rinde, welche das Mark wie ein Mantel bedeckt und nur von einzelnen Trabekeln samt begleitenden Sinus durchzogen wird. Die echten Sekundärknötchen liegen stets nur in einfacher Lage in der äußersten Schicht der Rinde (Abb. 3), und nicht in der Mitte derselben, wie bisher angenommen wurde, und auch nicht in der Mitte der Pseudosekundärknötchen. Im Inneren der Rinde und in den Marksträngen treten sie nur unter pathologischen Bedingungen auf. Die Pseudosekundärknötchen nehmen hingegen meist die ganze Breite der Rinde ein und bilden knötchenförmige Verdickungen derselben. Unter normalen Bedingungen kommen echte Sekundärknötchen nur in ihrer an den Randsinus angrenzenden Schicht vor.

Im Bau des lymphatischen Gewebes finden sich nur in der Milz einige Unterschiede, die aber mehr die Anordnung als das Wesen dieses Gewebes betreffen. Hier fehlen die für das lymphoide Gewebe charakteristischen Venen. Auch scheinen eigentliche Pseudosekundärknötchen nicht vorzukommen. Das Fehlen der Venen muß wohl dadurch erklärt werden, daß hier das lymphatische Gewebe bereits in die Blutbahn eingeschaltet ist, so daß die Lymphocyten direkt in sie einwandern können, ohne besonderer Venen zu bedürfen. Das anscheinende Fehlen von Pseudosekundärknötchen in der Milz mag ähnliche Gründe haben.

Die FLEMMING'schen Sekundärknötchen des lymphatischen Gewebes der Darmschleimhaut sind bei den Herbivoren reich an Lipochrom.

ABSCHNITT II

Experimentelle Untersuchungen über die Lymphopoëse und über die Histogenese der Sekundärknötchen und ihre Bedeutung

Über die Entstehung der Lymphocyten in der Ontogenese sind sich fact alle Autoren einig (WEIDENREICH (1909), MAXIMOW (1927)).

Wie besonders ALFEJEW (1924) gezeigt hat, entstehen sie aus den embryonalen Mesenchymzellen zu einer Zeit, wo diese noch nicht differenziert sind und auch noch eigentliche Gitterfasern fehlen. Dabei wird das Protoplasma dieser Zellen stark basophil, werden ihre protoplasmatischen Ausläufer eingezogen und rundet sich der Zelleib ab. An diesem Prozeß nehmen die Mesenchymzellen sowohl des lymphoiden Gewebes als auch der Lymphsinus teil.

Die Ansicht GULLAND's (1894), daß die ersten Lymphocyten der Lymphknoten aus dem Blutstrom in diese einwanderten, hat sich nicht bestätigen lassen. Sein Argument, daß sie erst dann in den Lymphknoten aufträten, wenn sie bereits im Blute vorhanden wären, ist durch ALFEJEW (1924) widerlegt worden.

Mit dem Anwachsen der Lymphocytenzahl in den Lymphknoten treten hier nun die ersten Gitterfasern und Phagocyten auf. Es differenzieren sich mit anderen Worten jetzt auch die ersten typischen Reticuloendothelien aus dem embryonalen Mesenchym heraus. Sie bleiben im Gegensatz zu den Lymphocyten im engen Verbande mit den Fasern und lösen sich nicht ab. Sie besitzen einen blassen Kern, häufig azidophiles Protoplasma und phagozytieren.

Die undifferenzierten embryonalen Mesenchymzellen nehmen mit dem Wachstum der Lymphknoten und des Embryos immer mehr an Zahl ab.

Während soweit keine ersten Meinungsverschiedenheiten über die Lymphocytenbildung bestehen, gehen die Ansichten der Autoren betreffs der postfetalen Lymphopoëse weit auseinander. Die einen glauben, daß die Lymphocyten sich im weiteren Leben nur aus den im Fetalleben gebildeten Lymphocyten regenerieren, indem diese durch Teilung neue Lymphocyten bilden. Die anderen nehmen an, daß auch im postfetalen Leben eine ständige Neubildung aus fixen Zellen stattfindet.

Zu den Vertretern der ersten Anschauung gehören JÖST und EMSHOFF (1912), HELLY (1914), NÄGELI (1923, 1925), ASCHOFF (1924, 1926) und seine Schüler KIYONO (1914), UCHINO (1925 a), MASUGI (1927) und KARMALLY (1929). Sie gründen ihre Anschauung besonders auf das unterschiedliche Verhalten der Lymphocyten und Reticuloendothelien bei Speicherung und Phagocytose und ferner auf das Fehlen von Übergangsbildern zwischen diesen beiden Zellen.

Dabei haben diese Autoren die undifferenzierten Mesenchymzellen fast gar nicht berücksichtigt. Nur NÄGELI (1923) und KARMALLY (1929) haben sie nebenbei erwähnt, ohne ihnen irgendeine Wichtigkeit zuzuschreiben.

Die undifferenzierten Mesenchymzellen sind nun aber zweifellos die Zellen, welche die meisten von jenen Autoren gemeint haben, die sich für eine Entstehung der Lymphocyten aus fixen Zellen ausgesprochen haben. Ich meine damit ZIEGLER (1889), WEIDENREICH (1909), DOWNEY und WEIDENREICH (1912), GREGGIO (1913), HERZOG (1923), PETRI (1925), CUNNINGHAM, SABIN und DOAN (1925) und ROTTER (1927). Alle diese Autoren haben aber diese Zellen nicht Genauer charakterisiert. Sie konnten sich unter diesen Zellen nichts genaues vorstellen und gründeten ihre Anschauung auf ein mehr theoretisches Erfordernis. Was MARCHAND (1913, 1924) als undifferenzierte Mesenchymzellen oder Adventitiazellen beschrieben hat, entspricht verschiedenen Zellen, nämlich unser undifferenzierten Mesenchymzellen und den eigentlichen Reticuloendothelien und Histiocyten.

Die undifferenzierten Mesenchymzellen sind zuerst von RIBBERT (1890) genauer beschrieben und abgebildet worden. Er hat unter den fixen Zellen des lymphatischen Gewebes zwei Arten unterschieden, nämlich „Reticulumzellen“, welche klein und unbedeutend erschienen, und „Endothelien“, die sich durch größere, helle, ovale oder rundliche Kerne auszeichneten. Beide Zellen fanden sich in den Lymphknoten sowohl im lymphatischen Gewebe wie in den Sinus. Aber erst MAXIMOW (1926, 1927) hat diesen beiden Zellen eine bestimmte Bedeutung beigelegt, indem er auf Grund seiner Untersuchungen zu dem Schluß kam, daß die Zellen, welche RIBBERT Reticulumzellen nannte, undifferenzierte Mesenchymzellen mit großen prospektiven Potenzen wären, während RIBBERT's Endothelien bereits mehr oder weniger einseitig ausdifferenzierte Elemente wären. Die letzteren entsprechen unseren Reticuloendothelien.

Die beiden Anschauungen über die Lymphopoëse stimmen, wie aus dem Gesagten hervorgeht, darin überein, daß die Reticuloendothelien schon mehr oder weniger weit ausdifferenzierte Elemente sind, welche noch Histiocyten und wohl auch noch Fibroblasten, aber nicht mehr Lymphocyten liefern können. Sie gehen insofern auseinander, als die eine Lehre, wie sie von ASCHOFF und seiner Schule vertreten wird, der Lymphocytenentstehung aus den undifferenzierten Mesenchym-

zellen keine besondere Bedeutung zumißt, während die andere Lehre, für die besonders MAXIMOW und seine Schule eingetreten sind, in den undifferenzierten Mesenchymzellen die eigentlichen Mutterzellen der Lymphocyten sieht.

Die meisten Autoren nehmen auch eine Neuentstehung von großen Lymphocyten aus kleinen an, indem sie glauben, daß die kleinen Lymphocyten durch Hypertrophie wieder zu großen mit besonderer Teilungsfähigkeit versehenen Zellen werden können.

Diese Anschauung ist für jene Autoren, die an eine alleinige Neubildung von Lymphocyten aus bereits vorhandenen glauben, ein theoretisches Erfordernis, da man oft große Lymphocyten in einem Gewebe auftreten sieht, das vorher gar keine großen oder mittelgroßen Lymphocyten enthielt. DOWNEY und WEIDENREICH (1912) haben für diese Theorie nur anführen können, daß man kleine Lymphocytenkerne von großem Protoplasmaleib umgeben fände und umgekehrt. Auch NÄGELI (1923), der auf eine solche rückläufige Umwandlung besonderes Gewicht legt, weiß keine weiteren Argumente hierfür anzuführen. Er widerspricht sich vielmehr, da sich nach seiner Auslegung kleine, mit dickem Chromatinnetz versehene, d. h. alte Lymphocyten wieder in große, junge, mit feinem Netz zurückverwandeln müßten.

Wie auch ROTTER (1927) betont hat, lassen sich für eine rückläufige Umwandlung der kleinen Lymphocyten in große keine Anhaltspunkte finden. Wir werden darauf bei der Besprechung unserer eigenen Experimente zurückkommen. Immerhin soll nicht geleugnet werden, daß kleine Lymphocyten unter Umständen wieder anschwellen können. Doch muß ein solcher Vorgang wohl ganz anders gedeutet werden und ist von ihm keineswegs bewiesen, daß er wieder zur Teilung führen kann.

Der Vollständigkeit halber muß hier noch erwähnt werden, daß einige Autoren (CUNNINGHAM, SABIN und DOAN (1925) und SEEMANN (1928, 1930)) zwischen die großen Lymphocyten und ihre fixen Mutterzellen eine freie runde „primitive Stammzelle“ einschieben, die alle weißen Blutzellen liefern soll. In den Lymphknoten soll sie aber im Gegensatz zum Knochenmark nur in spärlicher Zahl vorhanden sein, was sie nicht erklären können. Ich habe bei meinen Untersuchungen in Übereinstimmung mit anderen Autoren — die Primitivzelle anderer Autoren ist der große Lymphocyt—nichts von einer solchen Zelle gesehen. Da nun CUNNINGHAM, SABIN und DOAN diese Zellen vorwiegend in leukämischem Blut beobachtet haben, und auch SEEMANN angibt, daß diese Zellen nur unter pathologischen Bedingungen auftreten, liegt der Gedanke nahe, daß die Autoren ihre

Zellen falsch gedeutet haben und es sich vielmehr um pathologisch oder anderweitig veränderte Blutzellen gehandelt hat, auf deren Existenz besonders NÄGELI (1923) hingewiesen hat.

Während über die Lymphopoëse eine reichhaltige Literatur besteht, gibt es nur wenige Arbeiten über das Werden und Vergehen der Sekundärknötchen und ihre Beziehungen zum lymphoiden Gewebe. Auch hier ist die fetale Entwicklung relativ einfach und wohl ohne ernsthafte Meinungsverschiedenheiten. Wie wir durch verschiedene Untersuchungen wissen (KLING (1904) u. A.), bestehen die Lymphknoten zunächst aus einer von Kapsel und Randsinus umgebenen diffusen lymphoiden Masse, in welche die zentralen Sinus vom Hilus aus hinein wachsen. Sobald die Sinus dieses Gewebe aufgeteilt und den Randsinus überall erreicht haben (beim Menschen im 5.–6. Monat), treten, wie ich (1929 b) gezeigt habe, die ersten Sekundärknötchen in der Rinde auf. Ähnliche Verhältnisse finden sich in der Milz, wo zu Anfang des 5. Monats lymphoide Scheiden um die Arterien gebildet werden, in denen dann die ersten Sekundärknötchen nach meinen (1929 b) und ONO's (1930) Untersuchungen im 5.–6. Monat auftreten. Um die gleiche Zeit habe ich auch die ersten Sekundärknötchen in den Schleimhäuten beobachtet.

Alle die ersten Sekundärknötchen sind nun, wie ich früher gezeigt habe, solide und Pseudosekundärknötchen, die sich von denen im späteren Lebensalter in nichts unterscheiden. Zwischen ihnen beiden finden sich alle Übergänge, so daß es keinem Zweifel unterliegen kann, daß hier die letzteren aus den ersteren hervorgehen, indem diese wachsen, reicher an Gitterfasern werden und charakteristische Venen erhalten. Da sich aber schon im embryonalen Leben meist mehr solide als Pseudosekundärknötchen finden, müssen wir annehmen, daß sie nicht alle zu Pseudosekundärknötchen werden, sondern sich vorher im diffusen lymphoiden Gewebe auflösen können.

Auch im postfetalen Leben werden ständig solide Sekundärknötchen neugebildet, wie man sich an fast jedem Lymphknotenschnitt überzeugen kann. Auch hier entstehen sie selbständig im lymphoiden Gewebe, da sie die kleinsten Sekundärknötchen sind und man ihre Entstehung von wenigen Zellen an verfolgen kann.

Nur GROLL und KRAMPF (1920/21) und WAETJEN (1925) haben angenommen, daß die soliden Sekundärknötchen aus FLEMMING'schen hervorgehen und gewisser-

maßen ihre Ruhephase vorstellen. Wie ich schon früher ausgeführt habe, lassen sich dafür keine Stützen gewinnen. Neben der Tatsache, daß die soliden Sekundärknötchen die entwicklungsgeschichtlich jüngsten Sekundärknötchen sind, spricht vor allem ihre Größe gegen eine solche Auffassung, die für gewöhnlich weit hinter derjenigen der FLEMMING'schen Sekundärknötchen zurücktritt. Wir werden hierauf auf Grund unserer eigenen Experimente zurückkommen.

Während des ganzen Lebens können die soliden Sekundärknötchen weiterwachsen und ähnlich, wie im embryonalen Leben, zu Pseudosekundärknötchen werden, wie man mit allen Übergangsbildern belegen kann, während andere sich direkt im lymphoiden Gewebe auflösen.

Die Histologie des lymphatischen Gewebes und seine Genese wird im postfetalen Leben nun dadurch kompliziert, daß die FLEMMING'schen Sekundärknötchen oder die sog. „Keimzentren" und die Übergangssekundärknötchen dazukommen. Sie treten unter normalen Verhältnissen erst einige Zeit nach der Geburt auf, wie schon von GULLAND (1894) beobachtet. Wie ich schon früher (1929 a) in Übereinstimmung mit HELLMAN (1918/19, 1921) und HEIBERG (1923) ausgeführt habe, ist es nicht schwer zu erkennen, daß die Übergangszentren für gewöhnlich nichts weiter als sich regressiv umwandelnde FLEMMING'sche Sekundärknötchen sind. Die Frage, ob sie wieder zu echten soliden oder FLEMMING'schen Sekundärknötchen werden können, wie MAXIMOW (1927) angenommen hat, und ob man sie mit anderen Worten als Ruhephase den FLEMMING'schen Sekundärknötchen als einer aktiven Phase gegenüberstellen darf, war unentschieden.

Die FLEMMING'schen Sekundärknötchen entstehen teils aus den soliden Sekundärknötchen (HEIBERG (1923) und POL (1923)), teils auch außerhalb derselben im lymphoiden Gewebe. Über Einzelheiten ihrer Entstehung wußten wir wenig.

Wie schon früher mitgeteilt, findet man gelegentlich Übergänge von Übergangs- zu Pseudosekundärknötchen, während ich direkte Übergänge von FLEMMING'schen zu Pseudosekundärknötchen nicht gesehen hatte.

Im folgenden soll nun der Versuch gemacht werden, auf Grund von Experimenten tiefer in die Fragen nach der Entstehung der Lymphocyten und dem Werden und Vergehen der Sekundärknötchen und ihrer Beziehung zueinander und zum lymphoiden Gewebe einzu-

dringen. Dabei wollen wir auch die Auswanderung der Lymphocyten aus dem lymphatischen Gewebe und ihr Auftreten im Blut genauer studieren. Im Anschluß daran wollen wir dann feststellen, was unsere Ergebnisse über die Bedeutung der verschiedenen Sekundärknötchen und des lymphoiden Gewebes aussagen.

Material und Methode

Auf Grund meiner früheren Untersuchungen schienen mir Versuche mit Regeneration und Hyperplasie von lymphatischem Gewebe zum Studium der obigen Fragen besonders geeignet. Da wir nun wachsende Lymphknoten nicht direkt unter dem Mikroskop beobachten können, sondern auf Zeitserienuntersuchungen angewiesen sind, erwies es sich als notwendig, möglichst gleichartige Versuchsbedingungen zu schaffen. Lebende Bakterien, welche ich früher als Reizmittel benutzt hatte, erwiesen sich dabei als unzulänglich, weil das Verhältnis zwischen Virulenz des Erregers und Resistenz des Wirtes und die davon abhängige Keimvermehrung zu variabel waren. Ich habe hier deshalb nicht lebende Reizmittel von konstanter Konzentration benutzt, und die Variabilität der Empfänglichkeit, wie in früheren Versuchen, dadurch herabzusetzen versucht, daß ich nur junge männliche Kaninchen von gleichem Alter und Gewicht (1500–1900 g) benutzte.

Als Reizmittel verwandte ich geeignete Dosen von Staphylokokkenvaccine, Benzol-Olivenöl, Terpentinöl und Arsen. Mit der Vaccine erzielte ich die kräftigste Hyperplasie. Benzol-Olivenöl verwandte ich in Anlehnung an die Arbeiten von SELLING (1911) und LIGNAC (1928). Während SELLING gezeigt hatte, daß große Dosen Benzol das lymphatische Gewebe weitgehend zerstören, hatte LIGNAC mit kleinen täglichen Dosen eine lymphatische Hyperplasie erzeugen können. Meine Versuche, bei denen eine einmalige größere Dose gegeben wurde, führten ebenfalls zu Hyperplasie. Terpentinöl wählte ich wegen seiner stark Abszeß bildenden Eigenschaft. Ich erzielte damit in Übereinstimmung mit KIYONO (1914) ähnliche Ergebnisse wie mit Benzol. Arsen benutzte ich schließlich auf Grund von WÄTJEN's Versuchen (1925), wobei ich seine mit tödlichen Dosen ausgeführten Versuche dahin erweiterte, daß ich bei den Tieren vor den Arseneinspritzungen durch Staphylokokkenvaccinebehandlung auch in den peripheren Lymphknoten und in der Milz echte FLEMMING'sche Sekundärknötchen erzeugte. Außerdem habe ich kleine nicht tödliche Dosen angewandt und nach anfänglichem Lymphocytenzerfall eine Neubildung und Regeneration beobachtet.

Die Reizmittel wurden subkutan in den rechten Unterschenkel der Kaninchen eingespritzt, da dieses Gebiet nur einen regionären Popliteallymphknoten hat, der sich unter normalen Bedingungen im Ruhezustand befindet und nur selten echte FLEMMING'sche Sekundärknötchen besitzt.

Bei allen Serien, bei welchen ich Hyperplasie und Regeneration studierte, habe ich auch das weiße Blutbild untersucht. Ich habe dabei neben Ausstrichen

auch die sog. Supravitalfärbung mit Neutralrot und Janusgrün angewandt, um die Monocyten sicher zu erkennen. In früheren Versuchen (1929 c und d), bei welchen ich diese Methode noch nicht benutzt habe, sind mir beim Ausdifferenzieren von Lympho- und Monocyten vielleicht Fehler zuungunsten der Lymphocyten unterlaufen, wobei die damals gezogenen Schlußfolgerungen aber bestehen bleiben müssen, wie sich aus diesen Arbeiten ergibt.

Bei allen Versuchsserien dieses Abschnitts wurden beide Popliteallymphknoten gewogen, und diese wie die Milz histologisch untersucht.

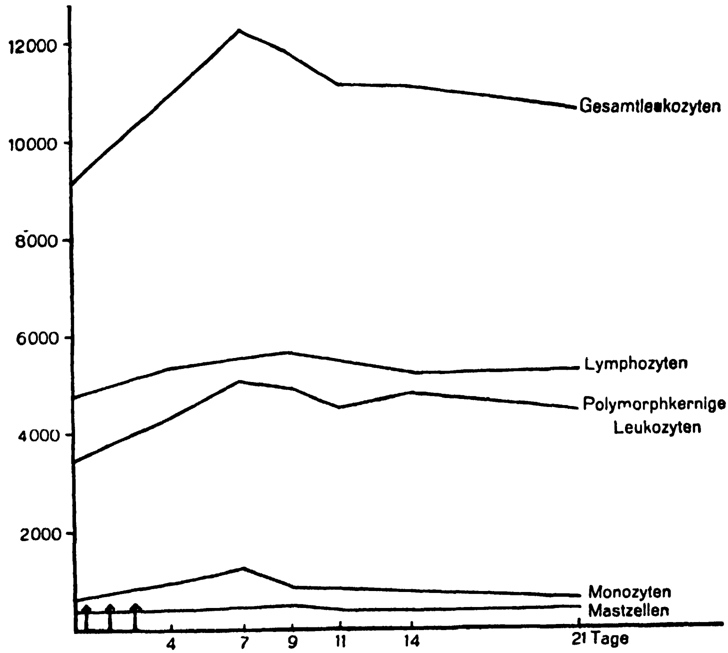

Abb. 23. Durchschnittliche Leukocytenkurven nach subkutaner Injektion von Staphylekokken vaccine (↑).

DIE EXPERIMENTE UND IHRE ERGEBNISSE

1. *Experiment: Versuche mit Staphylokokkenvaccine*

36 Kaninchen erhielten an 3 aufeinander folgenden Tagen je 1,0 ccm Staphylokokkenvaccine subkutan in den rechten Unterschenkel. Die Vaccine war dadurch gewonnen worden, daß 20 stündige gut gewachsene Bouillonkultur 2 Stunden auf 60° erhitzt, die Flüssigkeit abzentrifugiert und das Sediment mit physiologischer Kochsalzlösung wieder soweit verdünnt wurde, daß 1 ccm Vaccine 40 ccm Bouillonkultur entsprachen. Die Sterilität der Vaccine wurde einige Tage nach Herstellung geprüft.

Bei 14 Tieren wurde in bestimmten Abständen das weiße Blutbild bestimmt. Da sich 3 von diesen Tieren und ein weiteres 4. Tier bei der Sektion als schwer krank erwiesen (schwere Echinicoccuserkrankung, hochgradige Coccidiose u. a.), und ein 5. Tier während des Versuches starb, blieben nur II Tiere für Verwertung der Leukocytenzahlen und 31 Tiere zur histologischen Untersuchung übrig.

Resultate: Das weiße Blutbild

In Abb. 23 habe ich die durchschnittlichen Leukocytenkurven zusammengestellt. Wie ersichtlich ist, folgte auf die Injektion eine Monocytose, polymorphkernige Leukocytose und eine deutliche Lymphocytenvermehrung. Dabei lag der Gipfel der Lymphocytenkurve hinter dem der Monocyten und polymorphkernigen Leukocyten. Die Hauptvermehrung der Blutlymphocyten erfolgte in der ersten Woche des Experiments.

Makroskopische Untersuchung

An der Injektionsstelle fand sich zunächst eine hämorrhagischeitrige Entzündung. Am 4. Tage sah man einen etwa haselnuß- bis markstückgroßen Abszeß, der sich dann langsam verkleinerte. Am 14. Tag war er nur noch erbsen- und nach 21 Tagen ungefähr stecknadelkopfgroß.

Das Gewicht des rechten regionären Popliteallymphknotens vergrößerte sich in den ersten 4 Tagen aufs 4–6 fache, um dann langsam wieder abzunehmen (Abb. 24). Nach 3 Wochen war er durchschnittlich noch doppelt so schwer als normal. Am linken Popliteallymphknoten war keine deutliche Gewichtsvermehrung festzustellen.

Mikroskopische Untersuchung (Abb. 24)

a) Rechter Popliteallymphknoten

1 Tag nach der ersten Injektion waren die Sinus mit wechselnden Mengen polymorphkerniger Leukocyten und Erythrocyten gefüllt. Auch in den vorhandenen Übergangssekundärknötchen und den sinusnahen Teilen des lymphoiden Gewebes fanden sich polymorphkernige Leukocyten. Rinde und Markstränge waren von normaler Breite. Die Sekundärknötchen waren normal groß.

Die Veränderungen am lymphatischen Gewebe bestanden darin, daß überall große Lymphocyten neugebildet waren. Überall in den soliden und Übergangssekundärknötchen (Abb. 25 und 26) und in den sinusnahen Teilen des lymphoiden Gewebes wie auch in den Sinus

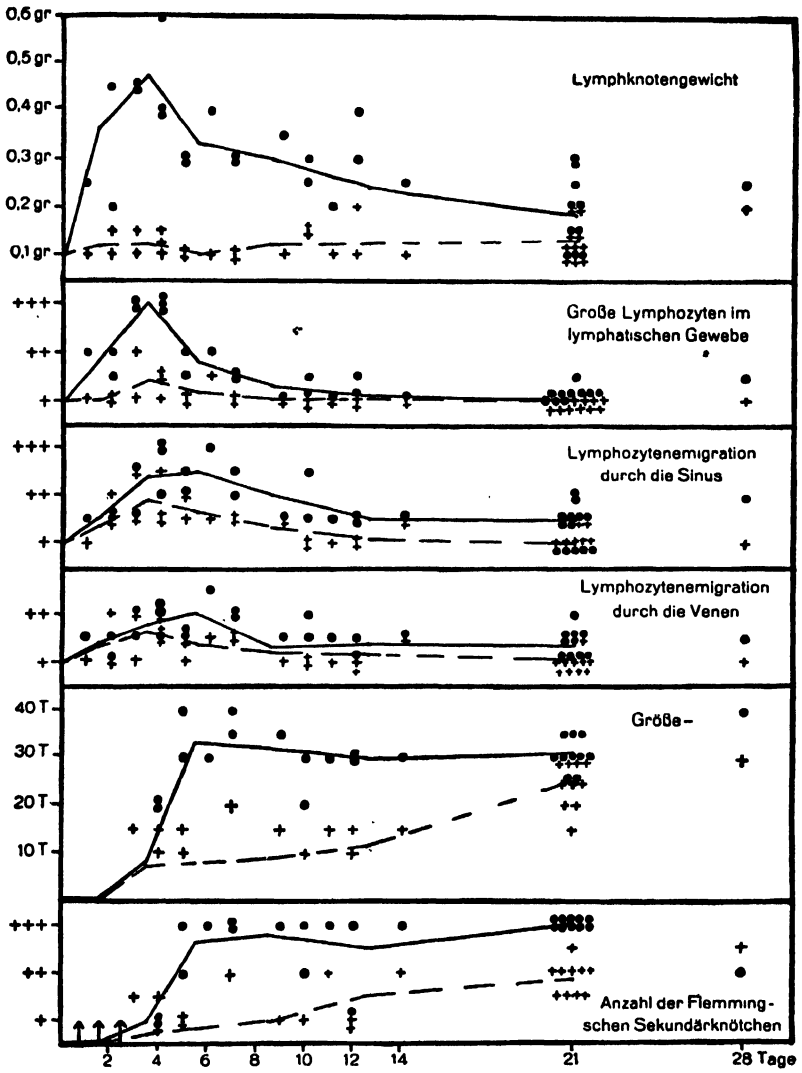

ABB. 24.[1]) Das Gewicht, die Zahl der großen Lymphocyten im lymphatischen Gewebe, die Stärke der Lymphocytenemigration und die Größe und Zahl der FLEMMING'schen Sekundärknötchen oder der sog. Keimzentren des rechten und linken Popliteallymphknotens nach subkutaner Injektion von Staphylokokkenvaccine in den rechten Unterschenkel (↑). •— rechter, +.... linker Lymphknoten.

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[1]) In dieser Abb. wie in Abb. 39, 42 und 44 habe ich die Zahl der großen Lymphocyten im lymphatischen Gewebe und die Stärke der Lymphocytenemigration

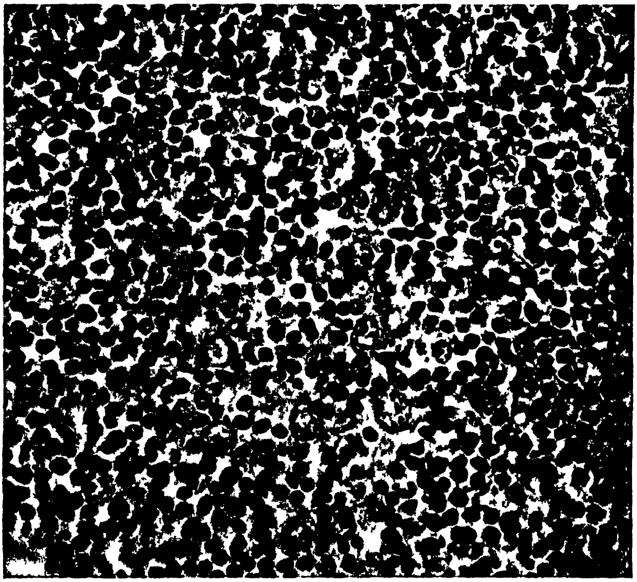

Abb. 25. Kan. 254. Rechter Popliteallymphknoten, 2 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 575 fache Vergr. Vermehrte große Lymphocyten in einem soliden Sekundarknötchen, z. T. mit Kernteilungsfiguren.

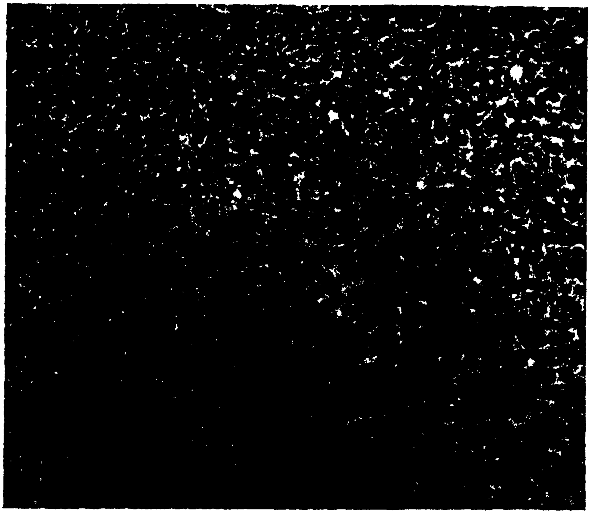

Abb. 26. Kan. 406. Rechter Popliteallymphknoten, 1 Tag nach der 1. Vaccineinjektion. Methylenblau-Eosin. 340 fache Vergr. Vermehrte große Lymphocyten in einem Übergangssekundärknötchen mit Kernteilungsfiguren. Daneben noch reichlich Kerntrümmer.

fanden sich vermehrte große Lymphocyten. Sie lagen besonders gern neben kleinen Blutgefäßen einzeln oder in kleinen Gruppen beieinander. Man sah deutliche Übergänge von undifferenzierten Mesenchymzellen (Taf. XI Abb. 1) zu großen Lymphocyten (Taf. XI Abb. 5), und zwar sowohl von denjenigen im lymphatischen Gewebe als auch von denen der Sinus. Man sah geschwollene Mesenchymzellen, deren Protoplasma stark basophil und deren Nukleolen vergrößert waren (Taf. XI Abb. 2). Dabei waren ihre protoplasmatischen Ausläufer, die sich bei der starken Basophilie ausgezeichnet verfolgen ließen, zunächst noch ausgestreckt. Dann rundeten sie sich aber ab (Taf. XI Abb. 3, 4). Die Zellen kontrahierten sich und wurden zu freien großen Lymphocyten mit allen Eigenschaften dieser Zellen. Der Kern schwoll gleichzeitig mit der ganzen Zelle an. Die eigentlichen schon ausdifferenzierten Reticulumzellen, welche RIBBERT (1890) als „Endothelien" bezeichnet hat, beteiligten sich nach meinen Beobachtungen nicht an der Lymphopoëse. Auch sie schwollen etwas, aber ohne daß ihr Protoplasma basophil wurde (Taf. XI Abb. 8). Sie begannen zu phagocytieren (Taf. XI Abb. 9). Dieses war besonders in den Übergangssekundärknötchen deutlich, die, wie ich im ersten Abschnitt beschrieben habe, besonders reich an schon ausdifferenzierten Reticulumzellen sind. Zwar wurden, wie schon erwähnt, auch hier überall große Lymphocyten neugebildet. Diese nahmen aber ihren Ausgang, wie im übrigen Gewebe, besonders entlang den Gefäßen von undifferenzierten Mesenchymzellen, während die größeren blassen Reticulumzellen noch vorhanden waren und sich mit Kerntrümmern und Leukocyten beladen hatten.

2 Tage nach der ersten Injektion waren Rinde und Markstränge deutlich verbreitert und die Sekundärknötchen vergrößert. Die Reticuloendothelien der Sinus waren stark vergrößert und vollgepfropft

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durch Vergleich der verschiedenen Präparate miteinander abgeschätzt. Die Maße der FLEMMING'schen Sekundärknötchen oder der sog. Keimzentren konnte ich hier nicht in der von HELLMAN und WHITE (1930) und SJOEVALL und SJOEVALL (1930) angegebenen Weise bestimmen, da zu meinen Versuchen ein viel zu großes Material nötig war. Ich habe mich deshalb darauf beschränkt, die Anzahl abzuschätzen und die Durchmesser der größten Keimzentren unter dem Mikroskop mit einem Okularmikrometer zu messen. Die in den Abb. hierfür angegebenen Zahlen beziehen sich auf Teilstriche. 1 Teilstrich = 0,015 mm.

mit polymorphkernigen Leukocyten und Erythrocyten. Sie waren aber nicht wesentlich vermehrt. Die meisten waren noch im Verbande. Ihre Kerne waren sehr groß geworden, wobei die Nukleolen die Größe derjenigen der großen Lymphocyten erreicht hatten.

Jetzt fanden sich überall in der Rinde und in den Marksträngen sehr reichlich große Lymphocyten. Man sah geradezu massenhaft Übergangsbilder von undifferenzierten Mesenchymzellen zu großen Lymphocyten. Auch hier waren wieder die gefäßnahen Bezirke bevorzugt. Ich habe 8–10 solcher Übergangsformen um einen Gefäßquerschnitt gesehen. In den kleinen Gefäßen wurde oft der Eindruck erweckt, als seien es die Endothelien selbst, welche sich zu Lymphocyten umbildeten. Das war aber zweifellos eine Täuschung, die dadurch hervorgerufen wurde, daß die Gefäßwände so dünn waren, daß die in der äußeren Schicht dieser Wände gelagerten Mutterzellen nach innen durchbrechen konnten. An größeren Gefäßen, wo Endothelien mit Sicherheit erkannt werden konnten, konnte ich einen solchen Vorgang nicht beobachten. Auch in diesem Stadium beteiligten sich nicht alle undifferenzierten Mesenchymzellen an der Lymphopoëse. Viele blieben klein und unscheinbar und ihr Protoplasma schwach azidophil. Sie blieben scheinbar ganz in Ruhe. Andere teilten sich vielleicht nur oder lieferten andere Blut- und Bindegewebszellen.

Die Veränderungen an den Sekundärknötchen am 2. Tage waren sehr einheitlicher Natur. Die normalerweise vorhandenen Übergangssekundärknötchen, die wir noch am vorigen Tage erkennen konnten, waren durch vergrößerte, vorwiegend aus kleinen Lymphocyten zusammengesetzte Knötchen ersetzt, denen mehr oder weniger reichlich große Lymphocyten beigemischt waren. Es hatte mit anderen Worten ein Wachstum der Übergangssekundärknötchen mit Bildung von kleinen Lymphocyten stattgefunden, dessen erste Anfänge wir im vorigen Stadium mit den vermehrten großen Lymphocyten festgestellt hatten. Die ursprünglich soliden Sekundärknötchen waren auch vergrößert und den anderen Sekundärknötchen ähnlich geworden. Daneben fanden sich auch kleine neugebildete solide Sekundärknötchen am äußeren Ende der Rinde. Von den größeren hier beschriebenen Sekundärknötchen konnte man z. T. nicht mehr mit Sicherheit sagen, ob sie noch echte oder schon Pseudosekundärknötchen waren.

Jene Sekundärknötchen aber, welche aus den Übergangssekundärknötchen erwachsen waren, muß man als Pseudosekundärknötchen bezeichnen, da sie ganz wie lymphoides Gewebe aussahen und auch Venen enthielten.

Am 3. und 4. Tage waren Rinde und Markstränge stark diffus verbreitert, und ließen sich eigentliche Sekundärknötchen außer einzel-

Abb. 27. Kan. 409. Rechter Popliteallymphknoten, 3 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 30 fache Vergr. Diffuse lymphoide Hyperplasie von Rinde und Marksträngen. Am Rande der Rinde einige Pseudosekundärknötchen.

nen soliden, am äußeren Rande der Rinde überhaupt nicht mehr deutlich erkennen (Abb. 27). Die Retikuloendothelien verhielten sich wie am vorigen Tage. Überall im lymphoiden Gewebe fanden sich jetzt massenhaft große Lymphocyten, besonders um Arterien (Abb. 28) und Venen (Abb. 29). Um die Gefäße herum bildeten sie oft dicke Mäntel. Es fanden sich noch reichlich Übergangsbilder aus

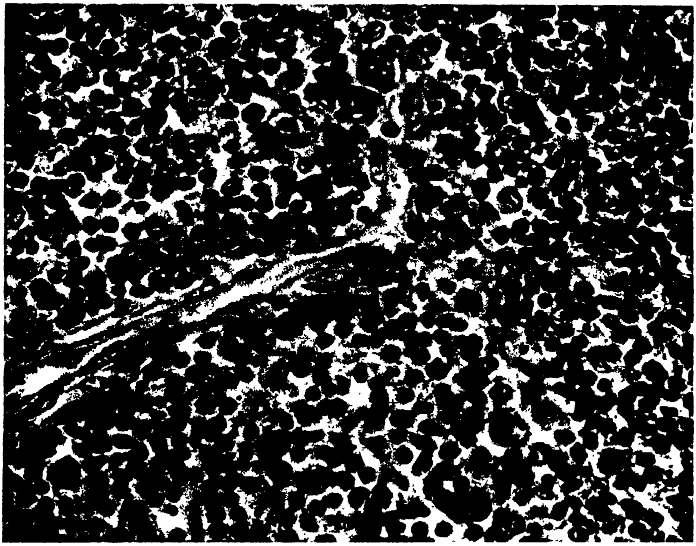

Abb. 28. Kan. 409. Wie Abb. 27. 575 fache Vergr. Neubildung von großen Lymphocyten um eine Arterie.

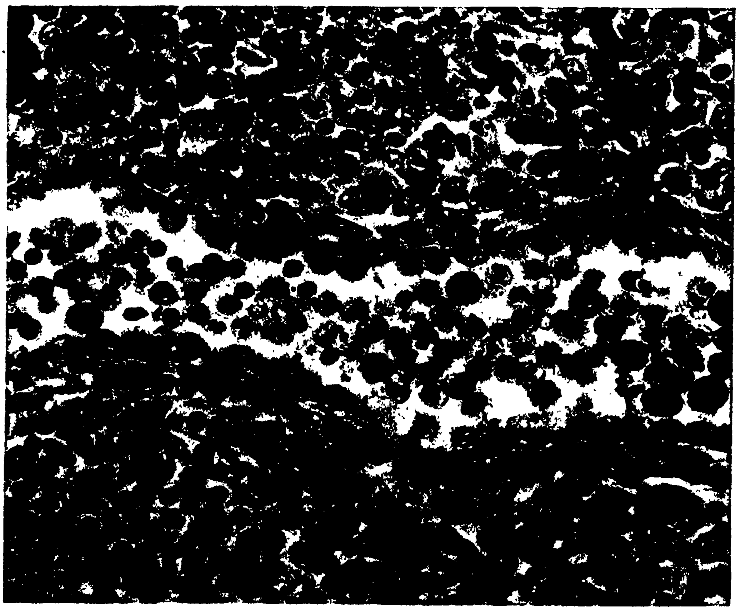

Abb. 29. Kan. 409. Wie Abb. 28. Neubildung von großen Lymphocyten Lymphocytenemigration. Im Lumen der Vene noch reichlich Leukocyten.

undifferenzierten Mesenchymzellen und sehr viele Übergänge von großen zu mittleren und kleinen Lymphocyten.

Die schon am 3. Tage deutlich vermehrte Lymphocytenemigration war am 4. Tage sehr stark geworden. Man sah überall in den Sinus massenhaft Lymphocyten (Abb. 30), die durch den Lymphstrom ins

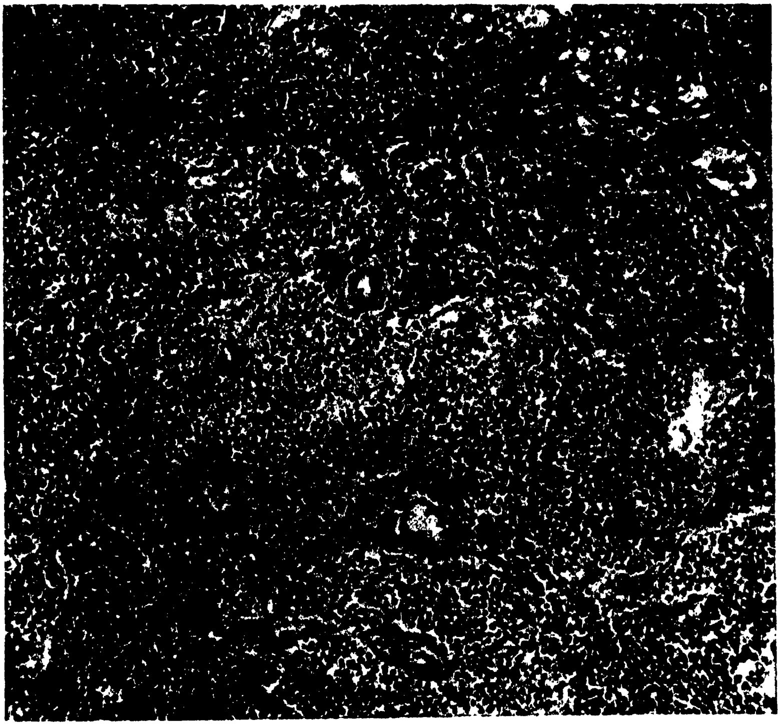

ABB. 30. Kan. 255. Rechter Popliteallymphknoten, 4 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 170 fache Vergr. Kräftige Lymphocytenemigration durch die Sinus.

Blut abgeführt wurden. Eine ebensolche Auswanderung fand direkt ins Blut durch die Venen des lymphoiden Gewebes statt (Abb. 31). Ihre Wände waren von zahlreichen Lymphocyten durchsetzt. Ihre Lumina waren damit gefüllt. Auch große Lymphocyten beteiligten sich an der Durchwanderung durch die Venenwände.

Am 5. und 6. Tage war die Hyperplasie zu ihrem Höhepunkt gekom-

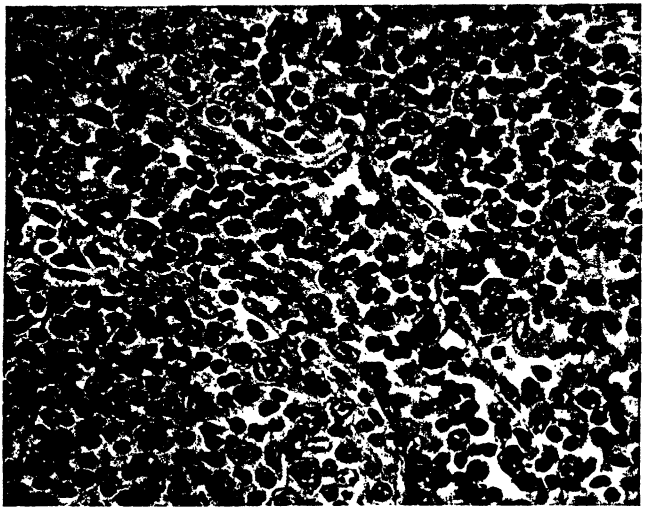

ABB. 31. Kan. 256. Rechter Popliteallymphknoten, 6 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 575 fache Vergr. Kräftige Lymphocytenemigration durch eine Vene. Um die Vene noch Neubildung von großen Lymphocyten mit allen Übergängen zu kleinen.

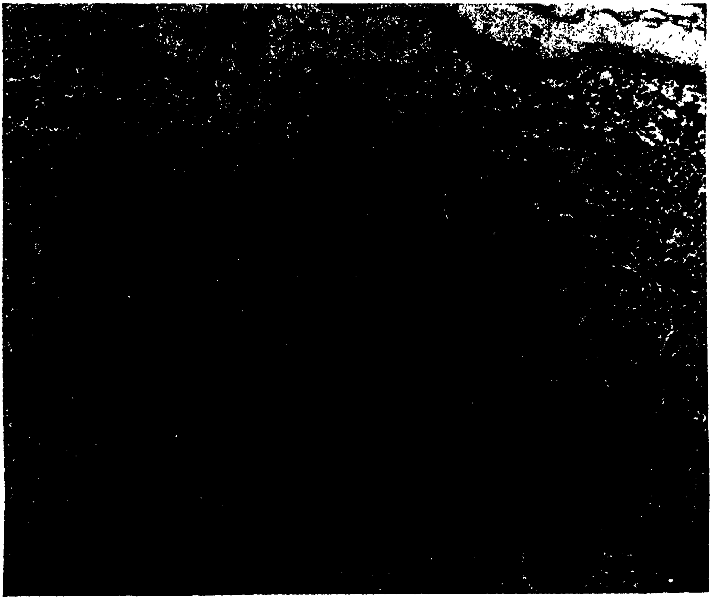

ABB. 32. Kan. 257. Rechter Popliteallymphknoten, 9 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 125 fache Vergr. Die Rinde enthält reichlich große FLEMMING'sche Sekundärknötchen oder sog. Keimzentren mit scharf begrenzten hellen Zentren.

men. Rinde und Markstränge hatten ihre größte Dicke erreicht. Die Sinus waren nur noch schmal und unbedeutend. Das lymphoide Gewebe enthielt an den meisten Stellen jetzt wesentlich verminderte große Lymphocyten und Übergangsbilder von undifferenzierten Mesenchymzellen.

Überall im diffusen lymphoiden Gewebe, besonders aber am Rande der Rinde sah man jetzt echte FLEMMING'sche Sekundärknötchen, von

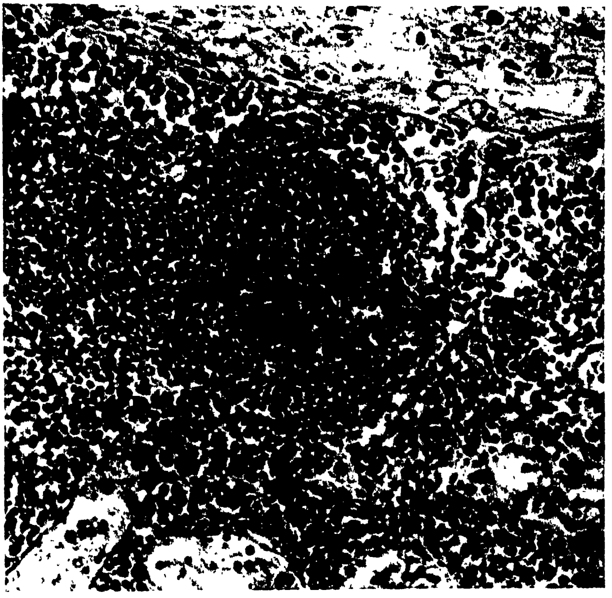

ABB. 33. Kan. 405. Rechter Popliteallymphknoten, 5 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 230 fache Vergr. Neubildung eines FLEMMING'schen Sekundärknötchens aus einem soliden. Das helle Zentrum ist noch nicht scharf von der Randzone abgegrenzt.

denen vereinzelte kleine schon bei einigen Tieren des 4. Tages sichtbar gewesen waren. Ihre Zahl und Größe erreichte ihren Höhepunkt am 6. Tage (Abb. 32). Bei den meisten Tieren behielten sie diese Ausdehnung bis zum Ende des Experiments bei. Die FLEMMING'schen Sekundärknötchen, welche wir am 5. Tage sahen, waren z. T. noch sehr klein (Abb. 33). Ihre Zentren waren alle typisch aus besonders mittelgroßen Lymphocyten zusammengesetzt, denen reichlich große

Lymphocyten beigemischt waren. Dort, wo Arteriolen und Kapillaren im Längsschnitt getroffen waren, konnte man die Entstehung der großen Lymphocyten aus den undifferenzierten Mesenchymzellen in allen Einzelheiten verfolgen. Je größer diese Sekundärknötchen

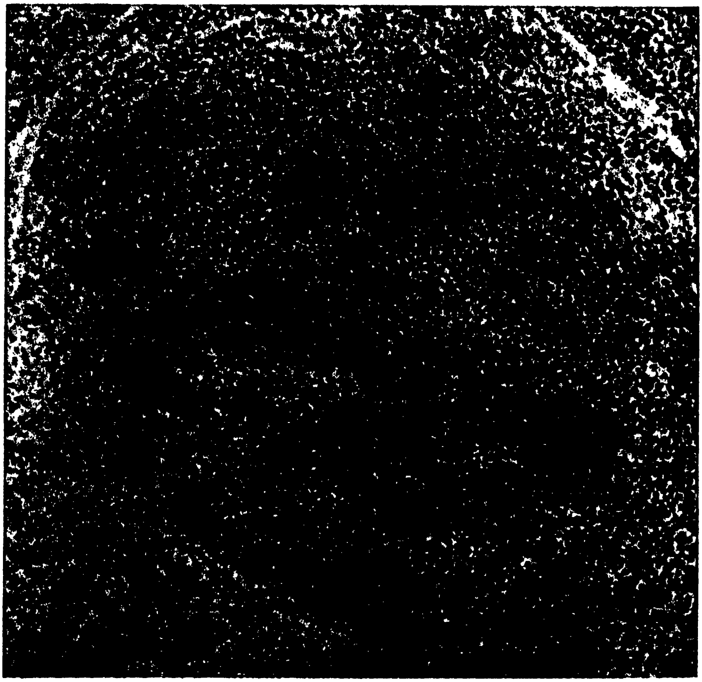

ABB. 34. Kan. 256. Rechter Popliteallymphknoten, 6 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 185fache Vergr. · Umwandlung der mittelgroßen Lymphocyten eines FLEMMING'schen Sekundärknötchens in kleine. Direkte Umwandlung eines FLEMMING'schen Sekundärknötchens in ein Pseudosekundärknötchen oder lymphoides Gewebe.

waren, um so schärfer waren sie meist vom lymphoiden Gewebe abgegrenzt.

Am Rande der Rinde traten die FLEMMING'schen Sekundärknötchen vorwiegend im Zentrum der soliden auf, und zwar bald mehr zentral und bald mehr der Eintrittsstelle der Arteriole zu, wodurch beim weiteren Wachstum des hellen Zentrums eine dieses ganz umgebende Randzone oder nur eine Kappe entstand. Im Inneren der Rinde und

in den Marksträngen entstanden die FLEMMING'schen Sekundärknötchen auch direkt im diffusen lymphoiden Gewebe, so daß dann eine Randzone oder Kappe fehlte. An der der Eintrittsstelle der Arteriole entgegengesetzten Seite war die Abgrenzung des hellen Zentrums in diesem Stadium oft unscharf. Man sah hier gelegentlich alle Übergänge von den mittelgroßen Lymphocyten zu den kleinen der Kappe oder Randzone. Bei einigen FLEMMING'schen Sekundärknötchen konnte man sogar auch eine direkte Umwandlung des ganzen hellen Zentrums in kleine Lymphocyten beobachten (Abb. 34). Man sah die großen und mittelgroßen Lymphocyten strangförmig von wohlgeformten kleinen Lymphocyten durchsetzt, mit denen sie durch alle Übergänge verbunden waren. Hieraus geht mit Sicherheit hervor, daß die mittelgroßen Lymphocyten der FLEMMING'schen Sekundärknötchen oder ihre hellen Zentren sich direkt, ohne erst Übergangszentren zu werden, in kleine Lymphocyten umwandeln können, eine Eigenschaft, die sie aber nur in diesen ersten Tagen ihrer Entwicklung aufwiesen und die später wieder ganz verloren ging. Es handelte sich hier um eine Zeit, wo sich die Tendenz der Lymphknoten, kleine Lymphocyten zu produzieren, mit der Tendenz überschnitt, die herdförmig angeordneten mittelgroßen Lymphocyten der FLEMMING'schen Sekundärknötchen zu erzeugen.

Die Lymphocytenemigration war in diesen Tagen fast ebenso stark wie am 4. Tage, wobei der durch die FLEMMING'schen Sekundärknötchen ausgeübte Wachstumsdruck wohl fördernd wirkte. Die Retikuloendothelien machten einen fast normalen Eindruck.

In den folgenden Tagen (vom 7. Tage ab) nahm das lymphatische Gewebe wieder an Gesamtmasse ab, und zwar auf Kosten des diffusen lymphoiden Gewebes. Die großen Lymphocyten und ihre Vorstufen wurden hierin immer seltener. Die Lymphocytenemigration ging stark zurück, um vom 8.–10. Tage an wieder normal zu sein.

Die FLEMMING'schen Sekundärknötchen blieben jedoch bis zum Ende des Versuches (bis zum 28. Tage nach der ersten Injektion) in voller Ausdehnung erhalten (Abb. 35). Dabei handelte es sich aber nicht um ein Stehenbleiben der einzelnen Knötchen auf der einmal erreichten Höhe, sondern um ein ständiges Vergehen und Neuentstehen derselben. Schon vom 7. Tage an fanden sich nämlich in einigen Sekundärknötchen regressive Veränderungen, welche besonders gerne

an der der Eintrittsstelle der Arteriole entgegengesetzten Seite begannen, während das andere Ende weiterwachsen konnte (Abb. 36). Gleichzeitig bildeten sich neue FLEMMING'sche Sekundärknötchen, die wieder zu großen heranwuchsen und immer schärfer begrenzt wurden. Ein Teil der Übergangssekundärknötchen oder regressiv veränderten FLEMMING'schen Sekundärknötchen behielt reichlich große Lympho-

ABB. 35. Kan. 528. Rechter Popliteallymphknoten, 21 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 100 fache Vergr. Die Rinde besteht fast ausschließlich aus FLEMMING'schen Sekundärknötchen mit scharf begrenzten hellen Zentren. Die Lymphocytenemigration hat aufgehört.

cyten und wuchs sich zu Pseudosekundärknötchen aus, wie sich an allen Übergangsbildern ablesen ließ. Sie hörten erst dann zu wachsen auf, wenn die großen Lymphocyten in ihnen verschwunden waren. Die ersten Pseudosekundärknötchen habe ich am 7. Tage gesehen, aber erst vom 10. Tage an waren sie scharf begrenzt. Nach der 2. Woche bestand das lymphatische Gewebe fast nur noch aus Sekundär-

knötchen, während das diffuse lymphoide Gewebe fast ganz verschwunden war (Abb. 35 und 37).

Nach der 3. Woche hörten bei einigen Tieren auch die Sekundärknötchen zu wachsen auf, indem die großen Lymphocyten aus ihnen verschwanden. Die Flemming'schen Sekundärknötchen wandelten sich mehr und mehr in Übergangssekundärknötchen um, welche sich diffus im lymphoiden Gewebe auflösten.

Abb. 36. Kan. 533. Rechter Popliteallymphknoten, 10 Tage nach der 1. Vaccineinjektion. Eisenhämatoxylin-Eosin. 125fache Vergr. Echtes Sekundärknötchen mit hellem Zentrum und Randzone. Während der distale, dem Randsinus zugelegene Teil des hellen Zentrums bereits in Auflösung begriffen ist, wächst der proximale Teil noch weiter.

b) Linker Popliteallymphknoten und Milz

Im linken Popliteallymphknoten, welchen ich in der gleichen Weise analysiert habe wie den rechten, spielten sich ganz ähnliche Vorgänge ab wie rechts (Abb. 24). Die Veränderungen waren jedoch nur geringfügiger Art. Sie waren denen im rechten Knoten parallel.

Ähnliche Veränderungen spielten sich auch im lymphatischen Gewebe der Milz ab. Hier habe ich allerdings nur die Entwicklung der Flemming'schen Sekundärknötchen verfolgt, welche in normalen Kaninchenmilzen fehlen. Sie traten

hier vom 4. Tage an auf und wurden bei den meisten Tieren bis zum Ende des Versuchs gefunden. Sie blieben aber an Größe und Zahl, wie diejenigen des linken Popliteallymphknotens, weit hinter denen des rechten Popliteallymphknotens zurück.

Zusammenfassung der Ergebnisse des 1. Experiments: Subkutane Injektionen von Staphylokokkenvaccine bestimmter Konzentration

ABB. 37. Kan. 588. Rechter Popliteallymphknoten, 21 Tage nach der 1. Vaccineinjektion. Methylenblau-Eosin. 30fache Vergr. Die Rinde besteht aus Pseudosekundärknötchen und FLEMMING'schen Sekundärknötchen. Die Lymphocytenemigration hat aufgehört.

an 3 aufeinander folgenden Tagen wurde von einer regionären Lymphdrüsenschwellung gefolgt, die in unserem Versuch 3–4 Tage nach der ersten Injektion ihren Höhepunkt erreichte und dann langsam wieder zurückging. Die Gewichtsvermehrung in den ersten Tagen war vorwiegend Folge einer exsudativen Lymphadenitis. Aber schon 1 Tag nach der ersten Injektion begann das lymphatische Gewebe zu wuchern. Es kam zunächst zu einer diffusen Lymphocytenneubildung überall in den Sekundärknötchen, im lymphoiden Gewebe und in den Sinus, die bis zum 4. Tag zu einer ausgedehnten diffusen lymphoiden

Hyperplasie führte. Die Lymphocyten nahmen ihren Ursprung von den undifferenzierten Mesenchymzellen, welche besonders entlang den Gefäßen angeordnet sind, sich aber auch sonst überall im lymphatischen Gewebe und in den Sinus finden. Dabei entstanden zunächst große Lymphocyten, die sich dann in mittelgroße und kleine umwandelten. Erst am 5. Tage traten überall echte FLEMMING'sche Sekundärknötchen auf, indem die Lymphopoëse sich mehr lokalisierte und bei der Bildung von mittelgroßen Lymphocyten stehen blieb. Mit dem Höhepunkt der Entwicklung der FLEMMING'schen Sekundärknötchen am 6. Tag erreichte auch die lymphatische Hyperplasie ihren Höhepunkt.

Bereits am 2. und 3. Tage fand sich eine vermehrte Lymphocytenemigration, welche ihren Höhepunkt am 4. Tage, also schon vor der Entstehung der FLEMMING'schen Sekundärknötchen erreichte. So blieb dann bestehen, bis die Sekundärknötchen voll ausgebildet waren (bis zum 7. Tage), und kehrte dann schnell zur Norm zurück. In der Zeit, wo wir die stärkste Lymphocytenauswanderung in den Lymphknoten sahen, fanden wir auch die stärkste Vermehrung der Lymphocyten im Blut.

Die FLEMMING'schen Sekundärknötchen blieben bis ans Ende des Versuches in voller Ausdehnung erhalten, indem sie ständig vergingen und neugebildet wurden. Nur in den ersten Tagen ihrer Entstehung, als sich auch noch eine starke Lymphocytenemigration fand, wandelte sich ein kleiner Teil direkt in kleine Lymphocyten, d. h. in Pseudosekundärknötchen und lymphoides Gewebe um. Später geschah das nur noch auf dem Umwege über Übergangssekundärknötchen.

Der linke Popliteallymphknoten und das lymphatische Gewebe der Milz beteiligten sich bei den meisten Tieren nur in geringem Maße an diesen Vorgängen. Ihre Veränderungen gingen denen des rechten regionären Popliteallymphknoten parallel.

2. *Experiment: Versuche mit Benzol-Olivenöl*

34 Kaninchen erhielten eine einmalige Injektion von 3 ccm Benzol-Olivenöl āā subkutan in den rechten Unterschenkel. 3 von diesen Tieren mußten von der Verwertung ausgeschlossen werden, da sie während des Versuches anscheinend an Benzolvergiftung starben. Bei 8 der übrigen Tiere wurden die Leukocyten in bestimmten Zeitabständen gezählt. Da vom 7. Versuchstage an die Injektionsstelle häufig offen und infiziert gefunden wurde, habe ich die Serie in zwei Reihen

zerlegt, in eine mit geschlossenem und eine mit offenem Verlauf. Für die Reihe mit geschlossenem Verlauf waren für das weiße Blutbild 5 Tiere und für die histologische Untersuchung 19 Tiere verwertbar, für die mit offenem Verlauf 6 resp. 12 Tiere.

Resultate: Das weiße Blutbild

Während die Tiere mit offenem Verlauf sehr verschiedene Leukocytenwerte zeigten, reagierten diejenigen mit geschlossenem Verlauf sehr einheitlich. In Abb. 38 sind die durchschnittlichen Leukocytenkurven der Tiere mit geschlossenem Verlauf zusammengestellt. Wie ersichtlich ist, folgte auf die Injektion eine deutliche Monocytose und polymorphkernige Leukocytose, deren Gipfel am 5. und 8. Tag gefunden wurden. In der 2. Woche, also später als bei den Staphylokokkentieren, folgte eine Lymphocytenvermehrung, die am 14. Tag ihren Höhepunkt erreichte.

Makroskopische Untersuchung

An der Injektionsstelle fand sich bei den Tieren mit geschlossenem Verlauf zunächst ein fast den ganzen Unterschenkel einnehmendes Ödem, zu dem sich vom 2. Tage an eine zunehmende Eiterung gesellte. Diese erreichte vom 5.–8. Tage ihren Höhepunkt, um dann wieder zurückzugehen. Am Ende der 2. Woche fand sich nur noch eine geringfügige Eiterung. Das Gewicht des rechten regionären Popliteallymphknoten vergrößerte sich in der 1. Woche aufs 3–5 fache, um dann langsam wieder abzunehmen (Abb. 39). Nach 4 Wochen war es nur noch doppelt so groß wie normal.

Bei den Tieren mit offenem Verlauf fanden sich an der Injektionsstelle verschieden starke Eiterungen. Der rechte regionäre Popliteallymphknoten war bis aufs 15fache vergrößert.

Mikroskopische Untersuchung (Abb. 39)

A. Geschlossener Verlauf

a) Rechter Popliteallymphknoten

In den ersten 2 Tagen spielten sich die wesentlichen Veränderungen in den Sinus ab. Am 1. Tage fanden sich hier reichlich polymorphkernige Leukocyten und Erythrocyten und vermehrte und geschwollene Reticuloendothelien, die meist im Verbande waren und gelegentlich

phagocytiert hatten. Am 2. Tag waren die Sinus mit großen zusammhängenden Nestern von Reticuloendothelien gefüllt, die vielfach mit Erythrocyten und polymorphkernigen Leukocyten beladen waren.

Rinde und Markstränge waren in den ersten beiden Tagen normal breit. Die Sekundärknötchen waren teils solide und meist Übergangssekundärknötchen mit wechselnden Mengen retikulärer Zellen, kleinen, mittleren und einzelnen großen Lymphocyten. Sie enthielten oft reichlich Phagocyten mit sehr vielen Kerntrümmern. Die Übergangssekundärknötchen bildeten sich in den folgenden Tagen weiter zurück,

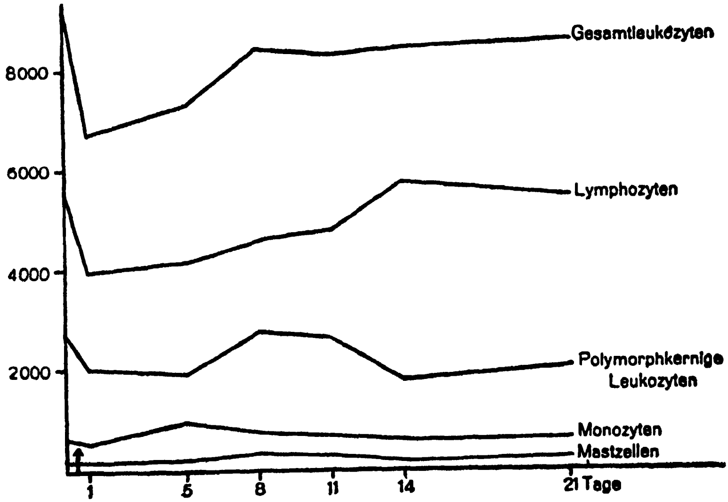

Abb. 38. Durchschnittliche Leukocytenkurven nach subkutaner Injektion von Benzol-Olivenöl (↑), geschlossener Verlauf.

indem die Reticulumzellen an Zahl zunahmen und die mittelgroßen und großen Lymphocyten immer spärlicher wurden. Andere lösten sich auch mit Bildung von kleinen Lymphocyten im lymphoiden Gewebe auf. Das lymphoide Gewebe war in diesen Tagen fast unverändert. Es bestand vorwiegend aus kleinen Lymphocyten mit spärlich großen Lymphocyten und aus Reticulumzellen, die wie in den Sinus und Sekundärknötchen vermehrt und vergrößert erschienen.

Erst am 3. Tage traten überall im lymphoiden Gewebe und in den Sekundärknötchen vermehrte große Lymphocyten auf, die hier in gleicher Weise wie beim 1. Experiment aus den undifferenzierten

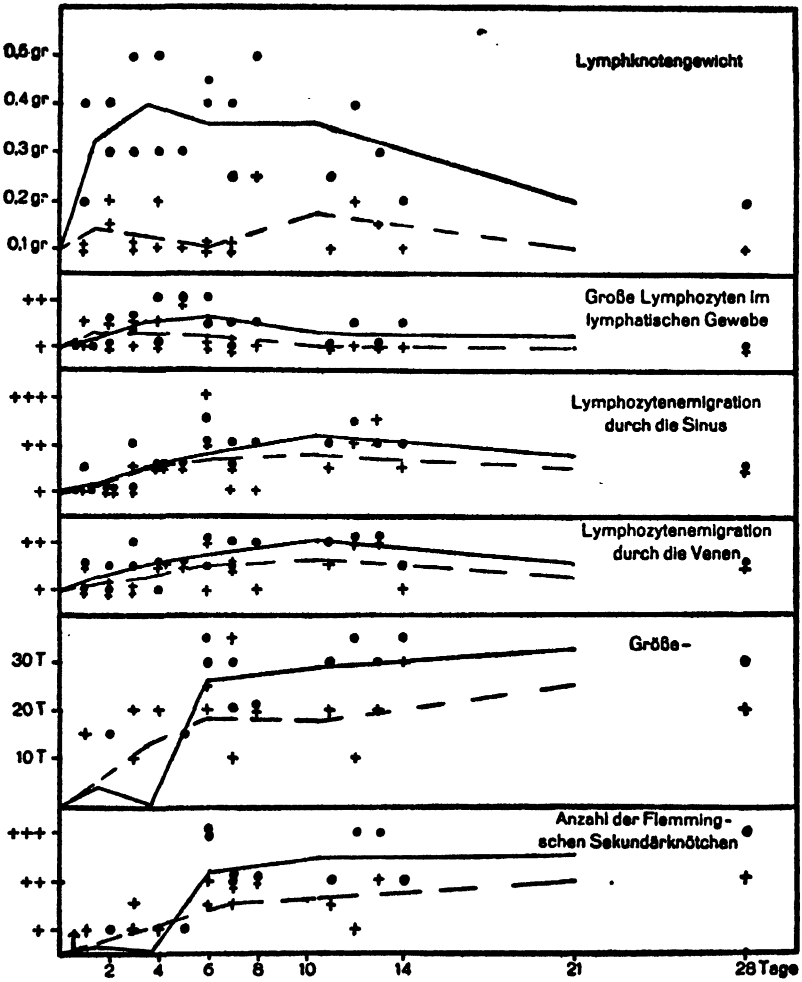

Abb. 39. Das Gewicht, die Zahl der großen Lymphocyten im lymphatischen Gewebe, die Stärke der Lymphocytenemigration und die Größe und Zahl der Flemming'schen Sekundärknötchen des rechten und linken Popliteallymphknotens nach subkutaner Injektion von Benzol-Olivenöl in den rechten Unterschenkel (↑), geschlossener Verlauf. •— rechter, + ... linker Lymphknoten.

Mesenchymzellen ihren Ursprung nahmen. Bei dem einen Tier dieses Tages war die Neubildung von großen Lymphocyten schon weiter fortgeschritten. In den Sinus habe ich hier aber, im Gegensatz zu den Staphylokokkentieren, keine Neubildung von Lymphocyten aus undifferenzierten Mesenchymzellen feststellen können, was wohl dadurch zu erklären ist, daß hier sehr viel mehr Reticuloendothelien neugebildet wurden als im 1. Experiment (vgl. Abb. 27 u. 40). Hier wurden die undifferenzierten Mesenchymzellen anscheinend zur Neubildung von Reticuloendothelien verbraucht oder überwogen die Reticuloendothelien bildenden Reize über die Lymphocyten bildenden.

Am 4. und 5. Tage waren Rinde und Markstränge deutlich verbreitert. Jetzt fanden sich überall im lymphoiden Gewebe und in den Sekundärknötchen sehr reichlich große Lymphocyten. Ihre Zahl blieb jedoch hinter der der Staphylokokkentiere zurück. In den Sinus und Venen konnte man eine deutlich vermehrte Lymphocytenemigration beobachten, die schon bei einem Tier des vorigen Tages deutlich gewesen waren. Die Reticuloendothelien waren weiter vermehrt und verdickt.

Am 6. und 7. Tage erreichten Rinde und Markstränge ihre größte Breite, die aber weit hinter derjenigen bei den Staphylokokkentieren zurückblieb. Die Verbreiterung war teils diffus, teils aber auch durch wohlbegrenzte Pseudosekundärknötchen hervorgerufen. Am 6. Tage fanden sich überall noch reichlich große Lymphocyten mit allen Übergängen zu mittelgroßen und kleinen. Am 7. Tage hatte die Menge der großen Lymphocyten jedoch deutlich abgenommen. Am Rande der Rinde zeigten sich jetzt FLEMMING'sche Sekundärknötchen in allen Größen. Stellenweise sah man junge Pseudosekundärknötchen mit noch reichlich großen Lymphocyten und typischen Venen. Die Lymphocytenemigration war jetzt kräftig geworden, doch nicht so stark wie im 1. Experiment. Die Reticuloendothelien der Sinus waren bei einigen Tieren zwar noch vermehrt, aber deutlich dünner geworden. Bei anderen Tieren waren sie gegen den 2. Tag unverändert (Abb. 40).

In den folgenden 8–14 Tagen wurde die Zahl der großen Lymphocyten im lymphoiden Gewebe immer geringer, um bei einigen Tieren schon am 9. Tage wieder normal zu sein. Rinde und Markstränge wurden auf Kosten des lymphoiden Gewebes wieder schmäler. Die

echten und Pseudosekundärknötchen blieben jedoch in voller Ausdehnung bestehen und bildeten sich genau so wie im 1. Experiment ineinander um. Man sah alle Übergänge von FLEMMING'schen Sekundärknötchen zu Übergangs- und von diesen zu Pseudosekundärknötchen. Daneben bildeten sich neue FLEMMING'sche Sekundär-

ABB. 40. Kan. 507. Rechter Popliteallymphknoten, 7 Tage nach der Benzol-Olivenöl-Injektion. Methylenblau-Eosin. 230 fache Vergr. Die Sinus sind mit Nestern von Uferzellen gefüllt, die überall im Verbande geblieben sind.

knötchen. Die Lymphocytenemigration dauerte zunächst an, um am 14. Tage dann deutlich abgenommen zu haben. Die Reticuloendothelien verhielten sich wie in den früheren Tagen. Selbst am 14. Tage konnten sie noch stark vermehrt und sehr dick sein.

Am 28. Tage waren schließlich die Sinus und das lymphoide Gewebe

wieder normal, während die FLEMMING'schen Sekundärknötchen unverändert weiterbestanden.

b) Linker Popliteallymphknoten und Milz

Auch hier kam es zu geringen Veränderungen, welche denen im rechten Popliteallymphknoten ähnlich waren und parallel gingen. Sie waren jedoch geringfügiger als bei den Staphylokokkentieren. In der Miiz fanden sich vom 6. Tage an bei etwa der Hälfte der Tiere echte FLEMMING'sche Sekundärknötchen.

B. Offener Verlauf

Da nicht genau festzustellen war, wann bei den Tieren dieser Reihe der Abszeß aufbrach und welche Erreger einwanderten, können wir sie nicht untereinander vergleichen. Es lohnt sich aber, Sektionsbefund und weißes Blutbild zu vergleichen und festzustellen, wodurch die geradezu enorme Lymphknotenhyperplasie dieser Lymphknoten bedingt war.

Die Hyperplasie dieser Lymphknoten, die in einigen Fällen das 15fache der Norm erreichte, war fast ausschließlich durch Neubildung von besonders FLEMMING'schen Sekundärknötchen bedingt, die vom 10. Tage an auch überall im Inneren der Rinde und Markstränge zu finden waren. Ihre Zahl und Größe übertraf diejenigen aller anderen von mir untersuchten Popliteallymphknoten. Das lymphoide Gewebe zeigte nur eine verhältnismäßig geringe Hyperplasie, die vom 21. Tage ab völlig zurücktrat. Sie erreichte nur die Ausdehnung der lymphoiden Hyperplasie der anderen Benzoltiere, nicht aber die viel stärkere der Staphylokokkentiere. Zu allen Zeiten fanden sich vermehrte große Lymphocyten im lymphoiden Gewebe, deren Zahl aber stets verhältnismäßig gering blieb. Gleichzeitig fand sich auch eine etwas vermehrte Lymphocytenemigration.

Bei 6 Tieren dieser Serie, bei welchen während des Experiments und am Todestage das weiße Blutbild bestimmt wurde, fand sich trotz der enormen Hyperplasie der FLEMMING'schen Sekundärknötchen bei keiner Zählung eine Lymphocytenvermehrung im Blut. Wir werden auf diesen Befund bei der Besprechung unserer Experimente zurückkommen.

Zusammenfassung der Ergebnisse des 2. Experiments: Auf subkutane Injektion von 3 ccm Benzol-Olivenöl āā in den rechten Unterschenkel von Kaninchen folgte eine Schwellung des rechten regionären Popliteallymphknotens, der in der 1. Woche das 3–5 fache seines Normalgewichts erreichte. Die Schwellung der ersten Tage war vorwiegend Folge einer exsudativen Lymphadenitis, wobei der reticuloendotheliale Apparat der Sinus aber viel stärker in Mitleidenschaft gezogen wurde und diese Veränderungen viel länger andauerten, als bei den Staphylokokkentieren. Hier war überhaupt die retikuläre

Reaktion viel stärker und die lymphocytäre geringer als im 1. Experiment. Das lymphatische Gewebe begann erst am 3. Tag zu wachsen. Es kam auch nur zu einer verhältnismäßig geringen Hyperplasie, die am 6.–7. Tage ihren Höhepunkt erreichte. Sie war auch nicht eine so diffuse, sondern es fanden sich stets wohlbegrenzte Pseudosekundärknötchen daneben. Die Auswanderung von Lymphocyten war in der 2. Woche am stärksten, also ebenfalls später als im 1. Experiment. Sie fand zu einer Zeit statt, wo die FLEMMING'schen Sekundärknötchen gerade ihre höchste Entwicklung erreichten. In der 2. Woche vermehrten sich auch die Blutlymphocyten am stärksten. Während dann in der 3. Woche die Lymphocytenemigration schnell nachließ und die Zahl der Blutlymphocyten wieder abnahm, blieben die FLEMMING'schen Sekundärknötchen wie im 1. Experiment bis ans Ende des Versuches in voller Ausdehnung bestehen, indem sie vergingen und neuentstanden, wie bei den Staphylokokkentieren beschrieben. Der linke Popliteallymphknoten und die Milz beteiligten sich nur in geringem Maße an diesen Vorgängen. Ihre Veränderungen gingen denen des rechten Popliteallymphknotens parallel.

Bei jenen Tieren, bei welchen die Injektionsstelle nicht geschlossen blieb und es zur Infektion kam, kam es zu einer hochgradigen lymphatischen Hyperplasie, wobei sich das Gewicht des regionären Lymphknotens aufs 15fache vermehrte. Die Hyperplasie war fast ausschließlich durch Neubildung von besonders FLEMMING'schen Sekundärknötchen bedingt, während das lymphoide Gewebe nur eine verhältnismäßig geringe Hyperplasie zeigte. Die Blutlymphocyten waren trotz der großen Anzahl und Größe der FLEMMING'schen Sekundärknötchen nicht vermehrt.

3. Experiment: Versuche mit Terpentinöl

54 Kaninchen erhielten eine einmalige subkutane Injektion von 0,15 ccm Terpentinöl in den rechten Unterschenkel. 5 Tiere starben während des Versuches und wurden nicht weiter verwertet. Bei 15 Tieren wurden die Leukocyten in bestimmten Zeitabständen gezählt. 6 davon mußten von der Verwertung ausgeschlossen werden, da sich 4 bei der Sektion als schwer krank erwiesen und bei 2 weiteren die Injektionsstelle offen und infiziert war. Von den übrigen 34 Kaninchen mußten noch 2 wegen schwerer Erkrankung und 5 wegen offener und infizierter Injektionsstelle ausgeschieden werden. Von den Tieren mit geschlossenem Verlauf konnten somit 9 Kaninchen für das weiße Blutbild und 36 für die histo-

logische Untersuchung verwandt werden. Die 7 gesunden Tiere mit offenem Verlauf wurden nur zum histologischen Vergleich herangezogen.

Resultate: Das weiße Blutbild

In Abb. 41 habe ich die durchschnittlichen Leukocytenkurven abgebildet. Auf die Injektion folgte auch hier eine deutliche Monocytose und polymorphkernige Leukocytose, die ihren Höhepunkt am 6. und 10. Tage erreichten. In der 2. Woche folgte dann eine Lympho-

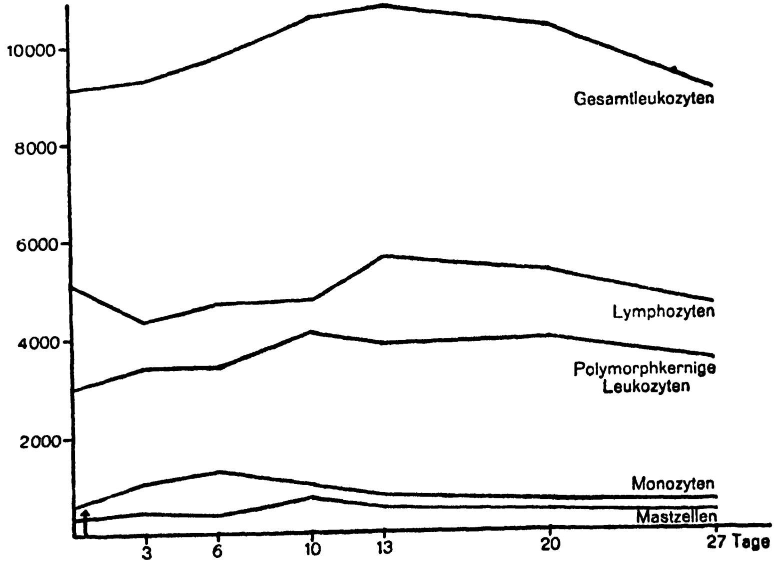

Abb. 41. Durchschnittliche Leukocytenkurven nach subkutaner Injektion von Terpentinöl (↑), geschlossener Verlauf.

cytenvermehrung, die ähnlich wie bei den Benzoltieren ihren Gipfel am 13. Tage erreichte.

Makroskopische Untersuchung

An der Injektionsstelle fand sich zunächst ein Ödem, das den größten Teil des Unterschenkels umfaßte und dem sich in den nächsten Tagen mehr und mehr Eiterzellen zugesellten. Am Ende der 1. Woche hatte die Eiterung ihren Höhepunkt erreicht und stellte jetzt einen Abszeß von ungefähr Fünfmarkstückgröße vor. Während sich der Abszeß bei einigen Tieren in der 2. Woche deutlich verkleinerte,

blieb er bei anderen Tieren bestehen. In der 3. Woche war er nur noch dreimarkstück- bis einpfennigstückgroß und am 27. Tage fand sich nur noch spärlich Eiter.

Der rechte regionäre Popliteallymphknoten vergrößerte sich zunehmend und erreichte sein größtes Gewicht am Ende der 1. Woche mit 3–4 facher Größe der Norm (Abb. 42). Selbst nach 27 Tagen war er bei den meisten Tieren noch 2–3 mal so schwer als normal.

Histologische Untersuchung (Abb. 42)

A. Geschlossener Verlauf

a) Rechter Popliteallymphknoten

Auch hier spielten sich in den ersten Tagen die wesentlichen Veränderungen in den Sinus ab. 1 Tag nach der Injektion fanden wir hier eine ausgedehnte eitrige Entzündung, wobei die Zahl der polymorphkernigen Leukocyten diejenige bei den beiden vorigen Experimenten noch übertraf. Freie polymorphkernige Leukocyten waren bis zum 6. und 7. Tage in den Sinus in größerer Zahl vorhanden. Am 2. Tage fand sich auch eine kräftige Schwellung und Vermehrung der Reticuloendothelien mit kräftiger Phagocytose. Rinde und Markstränge waren in den ersten 3 Tagen normal breit. Die Sekundärknötchen waren teils solide und meist Übergangssekundärknötchen mit wechselnden Mengen Reticulumzellen und kleinen, mittelgroßen und großen Lymphocyten. Sie enthielten oft reichlich Phagocyten mit Kerntrümmern. Auch hier entwickelten sich wie bei den Benzoltieren oft ganze Nester von Reticulumzellen. Andere Sekundärknötchen lösten sich mit Bildung von kleinen Lymphocyten im lymphoiden Gewebe auf.

Erst am 4. und 5. Tage waren Rinde und Markstränge deutlich verbreitert. Jetzt fanden sich auch deutlich vermehrte große Lymphocyten überall im lymphoiden Gewebe. Ihre Entstehung aus undifferenzierten Mesenchymzellen war deutlich nachzuweisen. Die Sinus waren anscheinend, wie bei den Benzoltieren, an der Lymphocytenbildung aus undifferenzierten Mesenchymzellen unbeteiligt. In diesen Tagen zeigten sich auch schon vereinzelte FLEMMING'sche Sekundärknötchen.

Am 6. und 7. Tage waren Rinde und Markstränge weiter verbreitert,

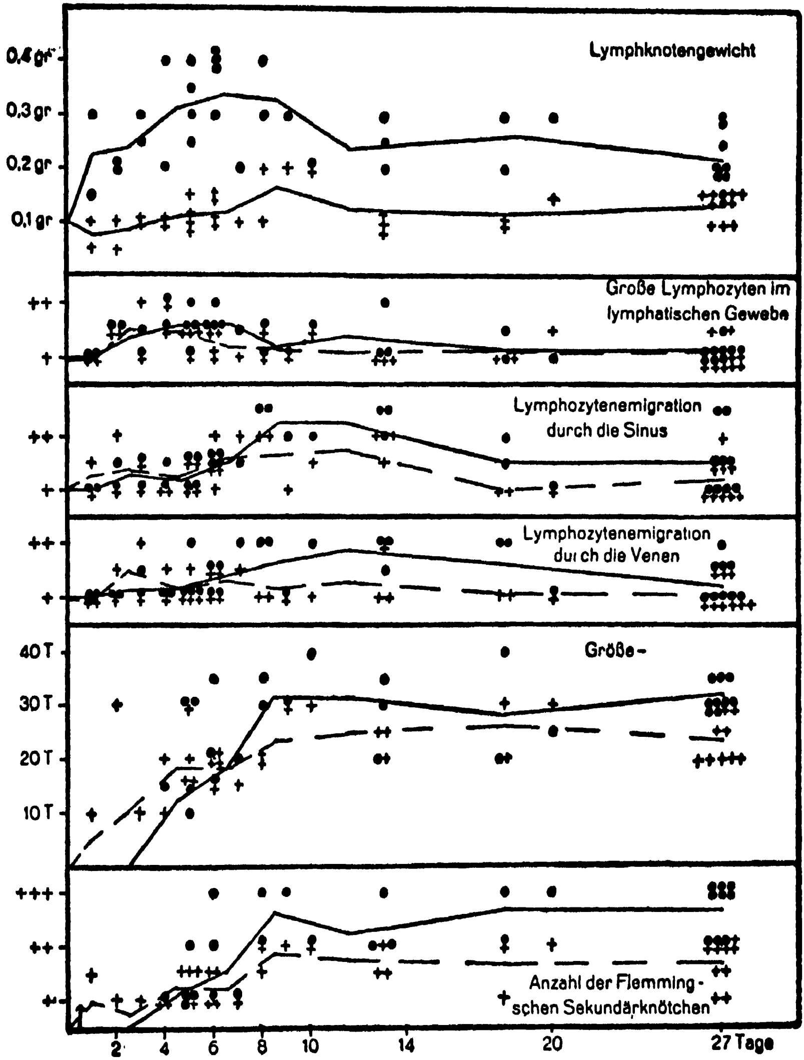

ABB. 42. Das Gewicht, die Zahl der großen Lymphocyten im lymphatischen Gewebe, die Stärke der Lymphocytenemigration und die Größe und Zahl der FLEMMING'schen Sekundärknötchen des rechten und linken Popliteallymphknotens nach subkutaner Injektion von Terpentinöl in den rechten Unterschenkel (↑), geschlossener Verlauf. •— rechter, + ... linker Lymphknoten.

ohne daß die Zahl der großen Lymphocyten wesentlich zugenommen hatte. Die Zahl der großen Lymphocyten blieb hinter der der Benzoltiere zurück. Die FLEMMING'schen Sekundärknötchen hatten an Zahl und Größe etwas zugenommen. Die Sinus waren noch sehr reich an Nestern geschwollener und vermehrter Reticuloendothelien.

Am Anfang der 2. Woche erreichte nun die lymphatische Hyperplasie ihren Höhepunkt, während die Menge der großen Lymphocyten im lymphoiden Gewebe deutlich abnahm. Jetzt fanden sich überall am Rande der Rinde und auch in den Marksträngen reichlich FLEMMING'sche Sekundärknötchen. In den Sinus und Venen sah man eine vermehrte Lymphocytenemigration, die im ganzen etwas schwächer als bei den Benzoltieren war. Die Reticuloendothelien bildeten sich bei einigen Tieren in dieser Woche zurück, während sie bei anderen bis zum Ende der Woche vermehrt und vergrößert waren.

In der 3. und 4. Woche ließen sich am lymphoiden Gewebe und an den Sinus keine Besonderheiten mehr erkennen. Die großen Lymphocyten waren normal an Zahl. Die Lymphocytenemigration hatte aufgehört. Die Reticuloendothelien hatten ihre normale Größe wieder erreicht, waren aber gelegentlich auch jetzt noch vergrößert und vermehrt. Die FLEMMING'schen Sekundärknötchen bestanden hingegen unverändert weiter.

b) Linker Popliteallymphknoten und Milz

Der linke Popliteallymphknoten nahm auch in diesem Experiment in geringem Maße an den beschriebenen Veränderungen teil (Abb. 42). Von der 2. Woche an fanden sich auch in der Milz häufig FLEMMING'sche Sekundärknötchen.

B. Offener Verlauf

Auch hier kam es, wie bei den korrespondierenden Tieren des Benzolexperiments, zu einer hochgradigen Hyperplasie des rechten regionären Popliteallymphknotens, dessen Gewicht sich bis aufs 9 fache vergrößerte. Die Hyperplasie war fast ausschließlich durch eine Vermehrung besonders der FLEMMING'schen Sekundärknötchen bedingt, während sich das lymphoide Gewebe nur wenig daran beteiligte. Die Lymphocytenemigration war nur wenig gegen die Norm vermehrt.

Zusammenfassung der Ergebnisse des 3. Experiments: Auf subkutane Injektion von 0,15 ccm Terpentinöl in den rechten Unterschenkel folgte bei unseren Tieren eine regionäre Lymphdrüsenschwellung. Der rechte Popliteallymphknoten schwoll in der 1. Woche auf das

3–4 fache seines Normalgewichts. Die Schwellung der ersten Tage war Folge einer eitrigen Lymphadenitis, die hier besonders heftig war und den reticuloendothelialen Apparat, wie bei den Benzoltieren, viel heftiger in Mitleidenschaft zog, als bei den Staphylokokkentieren. Das lymphatische Gewebe reagierte erst am 4. und 5. Tage mit einer Hyperplasie, die am 8. Tage ihren Höhepunkt erreichte und fast so stark war wie bei den Benzoltieren. Auch hier handelte es sich nicht um eine diffuse lymphoide Hyperplasie, sondern es fanden sich stets wohlbegrenzte Pseudosekundärknötchen daneben. Die Lymphocytenemigration war vom 8.–13. Tage am stärksten, womit auch die Lymphocytenvermehrung im Blut parallel ging. FLEMMING'sche Sekundärknötchen fanden sich vom 4. und 5. Tage an. Sie erreichten ihre größte Anzahl und Ausdehnung aber erst in der 2. Woche. Während dann in der 3. Woche die Lymphocytenemigration schnell nachließ und sich auch die Zahl der Blutlymphocyten wieder verminderte, blieben die FLEMMING'schen Sekundärknötchen, wie bei den beiden vorigen Versuchen, bis ans Ende des Experimentes in voller Ausdehnung erhalten, indem sie vergingen und neu entstanden, wie oben beschrieben. Der linke Popliteallymphknoten und die Milz beteiligten sich nur in geringem Maße an diesen Vorgängen. Ihre Veränderungen gingen denen des rechten Popliteallymphknotens parallel.

Bei den Tieren mit offener und infizierter Injektionsstelle kam es, wie bei den korrespondierenden Tieren des Benzolexperiments, zu einer hochgradigen lymphatischen Hyperplasie des regionären Popliteallymphknotens, die ebenfalls fast ausschließlich durch eine Neubildung von vorwiegend FLEMMING'schen Sekundärknötchen bedingt war. Auch hier fand sich nur eine gering vermehrte Lymphocytenemigration.

4. *Experiment: Versuche mit Arsen*

Zu diesen Versuchen wurde in Anlehnung an WAETJEN's Experimente (1925 a und b) eine 1% ige Lösung von Arsentrioxyd (MERK) benutzt, von der 73 Kaninchen verschiedene Dosen teils auf einmal, teils in aufeinander folgenden Tagen, teils intravenös, teils subkutan injiziert wurden. Hierbei zeigte es sich, daß einmalige subkutane wie intravenöse Injektionen von 0,01 g und mehr wie 2 oder mehrmalige Dosen von 0,005 g und mehr bei allen 24 so behandelten Tieren tödlich wirkten. 5 von diesen Tieren wurden am 3. und 4. Versuchstage getötet, während sie im Sterben lagen. Nur ein Tier überlebte eine Woche.

9 von diesen 24 Tieren habe ich mit 1,0 ccm abgetöteter Staphylokokkenbouillonkultur intravenös für eine Woche täglich vorbehandelt, um auch in ihren Milzen und peripheren Lymphknoten FLEMMING'sche Sekundärknötchen zu erzeugen (siehe meine Arbeit 1929 d), die bei normalen Kaninchen des hier benutzten Alters in diesen Organen für gewöhnlich fehlen. Sie erhielten ihre Arseninjektionen intravenös 2–4 Tage nach der letzten Vaccineinjektion. 4 weitere Kaninchen wurden als Kontrollen verwandt. Milz, Mesenterial-, Popliteal- und Cervikallymphknoten und der Blinddarm wurden histologisch untersucht.

Die übrigen 15 Tiere mit tödlichen Dosen bekamen ihr Arsen ohne Vorbehandlung subkutan in den rechten Unterschenkel. Bei ihnen wurde nur der regionäre Popliteallymphknoten genauer untersucht.

Alle diese Tiere stimmten darin mit denen von WAETJEN überein, daß sie der Arseninjektion erlagen. Sie zeigten jedoch insofern eine Verschiedenheit, als sie empfindlicher gegen Arsen waren als die von WAETJEN. Dieser Unterschied mag auf einer Verschiedenheit des benutzten Arsens beruhen — in seinen Arbeiten (1925) finden sich keine genauen Angaben über sein Präparat —, mag aber auch auf die Altersdifferenzen zwischen seinen und meinen Tieren zurückzuführen sein.

Jene 49 Tiere, welche eine einmalige Injektion von 0,001–0,005 g erhielten, blieben fast alle am Leben. Nur 5 von den 43, welche einmal 0,005 g erhalten hatten, starben während des Versuches. Bei allen diesen Tieren habe ich nur subkutan in den rechten Unterschenkel injiziert, um sicher zu sein, daß die zu untersuchenden Lymphknoten von dem Arsen getroffen wurden. Dieses wäre bei intravenöser Injektion wegen der durch den Blutstrom bedingten Verteilung schwer zu beurteilen gewesen. Die Tiere, welche 0,005 g erhielten, habe ich in ähnlicher Weise analysiert, wie die Tiere der ersten 3 Experimente. Sie interessierten hier besonders, weil WAETJEN bei kleinen Dosen eine Umwandlung von FLEMMING'schen in solide Sekundärknötchen beobachtet haben wollte, eine Beobachtung, welche mit dem, was wir bisher über die Sekundärknötchen erfahren haben, nicht übereinstimmt.

Bei der Besprechung der Resultate wollen wir zunächst die Versuche mit großen tödlichen Dosen mitteilen. Sie sind sehr einheitlicher Natur und von denjenigen mit nicht tödlichen Dosen in ihrem Verlauf und Histologie verschieden.

RESULTATE

A. Versuche mit tödlichem Verlauf

1. Versuche mit vorheriger Staphylokokkenvaccinebehandlung

Die 4 Kontrollen dieses Versuches, welche an denselben Tagen nach der letzten Vaccineinjektion getötet wurden, an welchen die Arsentiere starben oder getötet wurden, zeigten ähnliche Bilder, wie die korrespondierenden Tiere meiner Arbeit 1929 d. Auch die peripheren Lymphknoten und die Milz zeigten schön ausgebildete FLEMMING'sche

Sekundärknötchen mit vielen Kernteilungsfiguren. Das lymphoide Gewebe war reich an großen und mittelgroßen Lymphocyten. Die Lymphocytenemigration war deutlich vermehrt.

Die durch die Arseninjektionen hervorgerufenen Veränderungen stimmten weitgehend mit den von WAETJEN (1925) beschriebenen überein. Bei den Tieren, welche die größte einmalige Arsendosis bekommen hatten und welche bereits in der 1. Stunde nach der Injektion gestorben waren, zeigten die vorhandenen FLEMMING'schen Sekundärknötchen in den untersuchten Organen alle Anzeichen einer regressiven Umwandlung. Die großen Lymphocyten und Kernteilungsfiguren waren hier, wie in den anderen Sekundärknötchen, im lymphoiden Gewebe und in den Sinus, deutlich vermindert. Die Lymphocyten zeigten z. T. regressive Kernveränderungen mit allen Übergängen zu tingiblen Körperchen, die etwas gegenüber der Norm vermehrt waren. Bilder von stärkerem Kernzerfall waren bei diesen Tieren noch nicht zu sehen. Überall in den Sekundärknötchen, im lymphoiden Gewebe und in den Sinus sah man vermehrte und geschwollene Reticuloendothelien, die gelegentlich tingible Körperchen aufgenommen hatten.

Bei jenen 6 Tieren, welche 0,02–0,025 g Arsen in 2–3 Tagen intravenös erhalten hatten, und welche am 3. Versuchstage starben oder getötet wurden, waren die beschriebenen Veränderungen weit vorgeschritten. Die großen und teilweise auch die mittelgroßen Lymphocyten waren fast ganz verschwunden. Überall fand sich jetzt ein starker Lymphocytenzerfall mit allen Übergängen zu tingiblen Körperchen, die jetzt besonders in den Sekundärknötchen in sehr reicher Zahl vorhanden waren. Die Reticuloendothelien hatten sich stark vermehrt und vergrößert und vielfach tingible Körperchen phagocytiert. Die früheren FLEMMING'schen Sekundärknötchen bestanden fast nur noch aus retikulären Elementen, was, wie auch WAETJEN beschrieben hat, besonders in den Darmlymphknoten deutlich war. Das lymphoide Gewebe war auffallend zellarm geworden.

2. *Versuche ohne vorherige Staphylokokkenvaccinebehandlung*

Die Veränderungen in den regionären Popliteallymphknoten dieser Tiere waren prinzipiell dieselben wie in den lymphatischen Organen der soeben beschriebenen Kaninchen. Je später nach den Injektionen

die Tiere zur Sektion kamen, um so weniger große und mittelgroße Lymphocyten waren zu finden und um so stärker war der Lymphocytenzerfall fortgeschritten. Auch hier waren die Sekundärknötchen bevorzugt. Es kam, wie bei den vorigen Tieren, zu einer kräftigen Proliferation der Reticuloendothelien, welche die Lymphocytenkerntrümmer phagocytierten. Bei dem nach 7 Tagen gestorbenen Tier fand sich eine hochgradige Atrophie des lymphatischen Gewebes. Es war sehr arm an Lymphocyten geworden. Sie waren ausschließlich

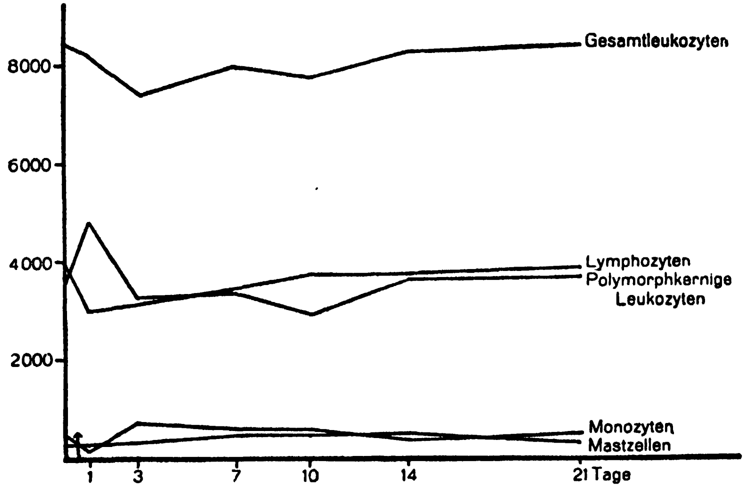

Abb. 43. Durchschnittliche Leukocytenkurven nach subkutaner Injektion von 0,005 g Arsen (↑).

nur noch kleine Lymphocyten. Irgendwelche Anzeichen von Neubildung fehlten.

B. Versuche mit nicht tödlichem Verlauf

1. Versuche mit 0,005 g Arsen

Das weiße Blutbild

Auf die Injektion folgte eine anfängliche Vermehrung der polymorphkernigen Leukocyten und Monocyten, während die Zahl der Lymphocyten sich zunächst verminderte (Abb. 43). Im weiteren Verlauf der 1. Woche kam es dann zu einer Lymphocytenregeneration, die am 10. Tage abgeschlossen war.

Makroskopische Untersuchung

An der Injektionsstelle fand sich außer geringen Nekrosen nichts Besonderes. Der rechte regionäre Popliteallymphknoten war 1 und 2 Tage nach der Injektion auf ungefähr das Doppelte vergrößert (Abb. 44), um dann wieder an Gewicht abzunehmen.

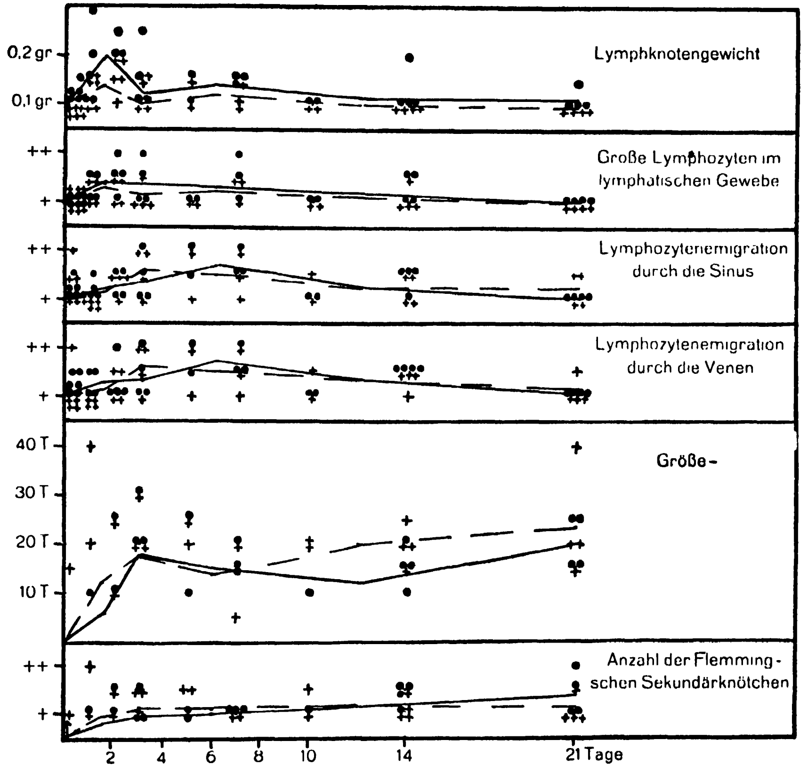

Abb. 44. Das Gewicht, die Zahl der großen Lymphocyten im lymphatischen Gewebe, die Stärke der Lymphocytenemigration und die Größe und Zahl Flemming'schen Sekundärknötchen des rechten und linken Popliteallymphknotens nach subkutaner Injektion von 0,005 g Arsen in den rechten Unterschenkel (↑). •— rechter, +.... linker Lymphknoten.

Mikroskopische Untersuchung (Abb. 44)

5–6 Stunden nach der Injektion fanden sich ähnliche Bilder wie bei den Tieren mit tödlichen Dosen. Man sah einen starken Lymphocytenzerfall, der besonders die großen und mittelgroßen Lymphocyten

betraf und in den Sekundärknötchen am stärksten war. Besonders hier fanden sich massenhaft tingible Körperchen und Schwellung und Wucherung der Reticuloendothelien, die vielfach Kerntrümmer phagocytiert hatten (Abb. 45). Nach ½ Tage waren die meisten tingiblen Körperchen bereits wieder verschwunden. Die Sekundärknötchen waren meist späte Übergangssekundärknötchen mit weiter

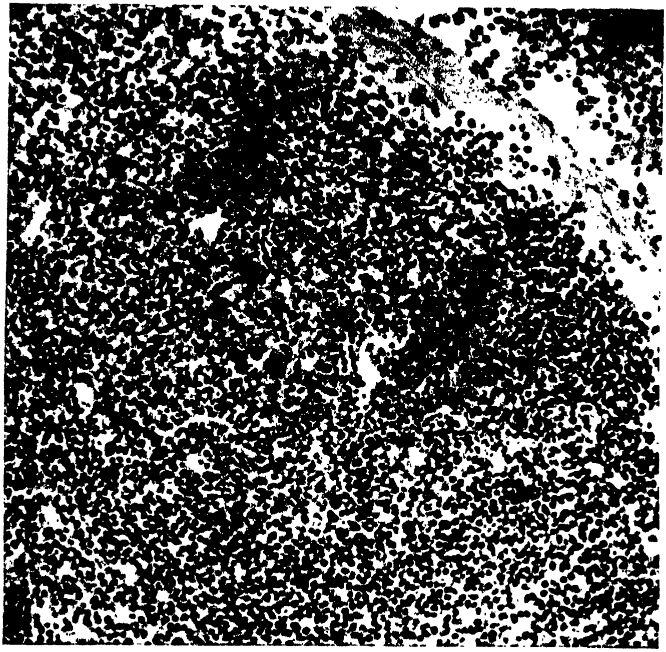

Abb. 45. Kan. 475. Rechter Popliteallymphknoten, 6 Stunden nach der Injektion von 0,005 g Arsen. Methylenblau-Eosin. 230 fache Vergr. Stark zerfallenes Sekundärknötchen mit massenhaft Kerntrümmern und reichlich Reticulumzellen.

vermehrten Reticuloendothelien. Während es nun oben bei den Tieren mit großen Dosen zu völligem Sistieren des Wachstums und Schwund der großen und mittelgroßen Lymphocyten kam, blieben hier von Anfang an stets einige große und mittelgroße Lymphocyten, z. T. mit Kernteilungsfiguren, erhalten. Schon 1 Tag, besonders aber erst 2 Tage nach der Injektion waren sie deutlich vermehrt, wobei

ihre Zahl aber weit hinter der der 3 ersten Experimente zurückblieb. Jetzt verbreiterten sich auch Rinde und Mark. Dabei entstanden überall am Rande der Rinde neue solide Sekundärknötchen, die zunächst im Gegensatz zu den späten Übergangssekundärknötchen sehr klein waren. Auch in den Übergangssekundärknötchen waren neue große Lymphocyten aufgetreten.

Erst vom 3. Tage an traten FLEMMING'sche Sekundärknötchen in Erscheinung und gleichzeitig eine vermehrte Lymphocytenemigration. Mark und Rinde erreichten ihre größte Breite, die sie etwa bis zum 6. Tage beibehielten. Der reticuloendotheliale Apparat bildete sich zur Norm zurück. Während nun die Lymphocytenemigration bereits am 10. Tage wieder zur Norm zurückgekehrt war, wo auch die Blutlymphocytenkurve ihren Gipfel erreicht hatte, blieben die FLEMMING'schen Sekundärknötchen bis zum 21. Tage, d. h. bis zum Ende des Versuches erhalten, indem sie vergingen und neuentstanden wie bei den 3 ersten Experimenten. Die hierbei entstandenen Übergangssekundärknötchen bildeten sich z. T. in Pseudosekundärknötchen um.

2. Versuche mit 0,001 und 0,0025 g Arsen

Die histologischen Bilder der regionären rechten Popliteallymphknoten dieser Tiere waren denen des vorhergehenden Versuches an den korrespondierenden Tagen so ähnlich, so daß ich sie hier nicht noch einmal zu besprechen brauche.

Zusammenfassung der Ergebnisse des 4. Experiments. Nach großen, tödlich wirkenden Dosen Arsen kam es bei unseren Kaninchen zum Stillstand der Lymphopoëse und regressiven Umwandlungen des lymphatischen Gewebes. Die großen und mittleren Lymphocyten wurden immer spärlicher, um schließlich ganz zu verschwinden. Bei dem Zerfall waren die großen, mittelgroßen und kleinen Lymphocyten in sich verringerndem Maße betroffen. Die Zelltrümmer wurden von den Reticuloendothelien aufgenommen, wobei sich diese stark vergrößerten und vermehrten. Die FLEMMING'schen Sekundärknötchen wandelten sich hierbei in Übergangssekundärknötchen und pathologische Formen um, die schließlich nur noch aus retikulären Elementen bestanden, wie besonders schön an den Darmlymphknötchen zu beobachten war. Eine Umwandlung in solide oder Pseudosekundärknötchen konnte nirgends beobachtet werden. Die

Vermehrung der kleinen Lymphocyten in den FLEMMING'schen und Übergangssekundärknötchen war nur eine scheinbare und nur eine Etappe auf dem Wege des Zerfalls der großen und mittelgroßen Lymphocyten in Kerntrümmer.

Nach kleinen, nicht tödlich wirkenden Dosen Arsen kam es in den regionären Lymphknoten zunächst zu einem starken Lymphocytenzerfall, der dem nach großen Dosen sehr ähnlich war. Hier folgte nun aber eine Lymphocytenneubildung, die schon 1 Tag nach der Injektion deutlich war und zu einer geringeren Hyperplasie des lymphatischen Gewebes führte. Eine Umwandlung von FLEMMING'schen zu soliden Sekundärknötchen ließ sich auch hier nicht beobachten. Die regressiv umgewandelten echten Sekundärknötchen lösten sich vielmehr diffus im lymphoiden Gewebe auf oder wuchsen zu Pseudosekundärknötchen aus.

Vom 3. Tage an sah man eine deutlich vermehrte Lymphocytenemigration, die bereits am 10. Tage zur Norm zurückkehrte. Hiermit ging eine Regeneration der Blutlymphocyten parallel. FLEMMING'sche Sekundärknötchen traten vom 3. Tage an in Erscheinung und blieben bis zum Ende des Versuches in voller Ausdehnung erhalten, indem sie, ähnlich wie bei den ersten 3 Experimenten, wieder vergingen und neugebildet wurden.

BESPRECHUNG DER VERSUCHSERGEBNISSE

1. Die Lymphopoëse

Durch Injektion verschiedener Reizmittel in den rechten Unterschenkel wurde bei unseren Kaninchen eine lymphatische Hyperplasie des rechten Popliteallymphknotens erzeugt, deren Entstehung bei allen 4 Experimenten weitgehend übereinstimmte. Eine gewisse Zeit nach der Injektion traten zunächst große Lymphocyten auf, die sich dann schnell vermehrten. Nur in ihrer Anzahl und dem Zeitpunkt ihres Auftretens fanden sich einige Unterschiede. Die größte Zahl dieser Zellen fand sich bei den Staphylokokkenvaccinetieren, eine kleinere bei den Benzol- und Terpentinöl- und die kleinste bei den Arsentieren.

Wie in den Protokollen beschrieben ist, entstanden diese ersten großen Lymphocyten direkt aus den undifferenzierten Mesenchymzellen,

indem das sonst kaum sichtbare Protoplasma dieser Zellen stark basophil wurde, und die Zellen anschwollen und sich mit Abrundung ihrer Ausläufer ablösten. Diese Lymphocytenentstehung war keineswegs auf einzelne Gebiete der Lymphknoten beschränkt, sondern war eine ganz diffuse. Ja selbst in den Sinus konnte ich wenigstens bei den Vaccinetieren eine wenn auch nur geringfügige Neubildung von großen Lymphocyten aus den zwischen den eigentlichen Uferzellen liegenden undifferenzierten Mesenchymzellen beobachten. Die stärkste Lymphopoëse aus undifferenzierten Mesenchymzellen wurde jedoch überall um die Blutgefäße herum sowohl im diffusen lymphoiden Gewebe als auch in den Sekundärknötchen beobachtet, wodurch die Angaben älterer Autoren (Flemming (1885), Gulland (1894), Sabin (1905), Marchand (1913), Greggio (1913), Herzog (1923), Orsós (1925 b), Rotter (1927), Nishikawa (1927) u. A.), daß die Lymphocyten besonders in Gefäßnähe entständen, bestätigt werden. Die größte Zahl solcher Ablösungen aus dem undifferenzierten Mesenchym fand sich gerade zu Anfang der Lymphocytenentstehung und zwar am meisten bei den Vaccinetieren, bei denen wir dann auch die größte Zahl großer Lymphocyten sahen.

Für eine Entstehung der ersten großen Lymphocyten aus kleinen fanden sich, wie zu erwarten war, keine Anhaltspunkte. Alle Befunde sprachen vielmehr dagegen. Übergangsbilder von großen zu mittelgroßen und kleinen Lymphocyten waren nämlich gerade zu Anfang meiner Versuche sehr spärlich. Die meisten großen Lymphocyten lagen anscheinend unabhängig neben den kleinen, ein Befund, der schon gelegentlich von Marchand (1913, 1924) und Maximow (1927) erhoben war. Die Übergangsbilder wurden vielmehr erst dann reichlich, wenn die großen Lymphocyten ihre größte Zahl erreicht hatten und die Zahl der Ablösungsbilder aus dem undifferenzierten Mesenchym stark zurückgegangen war. Alle diese Befunde können nur dahin gedeutet werden, daß die neuen großen Lymphocyten nicht durch Hypertrophie der kleinen und auch nicht durch Teilung der evtl. schon normalerweise vorhandenen großen Lymphocyten entstanden waren — denn normalerweise enthält das lymphoide Gewebe der Popliteallymphknoten fast überhaupt keine großen Lymphocyten —, sondern vielmehr direkt aus den undifferenzierten Mesenchymzellen.

Wie schon bemerkt, bildeten sich nicht alle undifferenzierten Mesen-

chymzellen in große Lymphocyten um. Einige blieben scheinbar ganz unbeteiligt, während sich andere wohl nur teilten und vielleicht auch andere Blut- oder Bindegewebszellen lieferten. Diese Beobachtung ist nicht verwunderlich. Denn würden sich alle Mesenchymzellen in der beschriebenen Weise aus dem Verbande lösen, würde ja das Gewebe an diesen Zellen verarmen und später bei einem eventuellen neuen Bedarf an großen Lymphocyten unfähig sein, solche zu produzieren.

Die großen und die später auch in großer Zahl vorhandenen mittelgroßen Lymphocyten vermehrten sich nun auch dann noch weiter, wenn die Zahl der Ablösungsbilder aus dem undifferenzierten Mesenchym bereits abgenommen hatte. Dabei teilten sich die großen und mittelgroßen Lymphocyten durch Mitose, wie sich durch zahlreiche Kernteilungsfiguren belegen ließ. Die häufigsten Mitosen fanden sich anscheinend in den großen Lymphocyten, die spärlichsten in den kleinen. Mit Abnahme der Größe der Zellen wurde also die Teilungsfähigkeit geringer.

Eine Umwandlung der eigentlichen Reticuloendothelien in Lymphocyten habe ich nirgends beobachten können, weder bei den Reticulumzellen noch bei den Uferzellen. Ich kann darin ASCHOFF und seinen Schülern nur beipflichten. Auch die Reticulumzellen der Sekundärknötchen nahmen keineswegs an der Lymphocytenbildung teil.

Da nun den Reticulumzellen der FLEMMING'schen und besonders der Übergangssekundärknötchen von manchen Autoren ein embryonaler Charakter zugeschrieben wird, muß ich hier noch etwas ausführlicher auf sie eingehen. HUECK (1920) sieht sie als ein besonders gutes Beispiel von undifferenzierten Mesenchymzellen an, aus denen Blutzellen entstehen sollen. MAXIMOW (1927) ist ähnlicher Ansicht. Wie aus seiner Abbildung 54 zu ersehen ist und wie ich auf Grund meiner Arbeiten nur bestätigen kann, handelt es sich da aber nicht um undifferenzierte Mesenchymzellen, sondern um dickere Zellen mit ausgedehntem meist azidophilem Protoplasma und großen hellen Kernen. Diese Zellen sind mit anderen Worten morphologisch mit unseren Reticulumzellen oder Reticuloendothelien identisch. MAXIMOW glaubte zwar gesehen zu haben, daß sie sich in Lymphocyten umwandeln können. Ich habe jedoch solche Bilder niemals gesehen. Auch glaube ich annehmen zu müssen, daß ASCHOFF und seine Schüler solche Übergänge vermißt haben und diese Beobachtung eine ihrer wesentlichen Stützen dafür ist, daß die Lymphocyten nicht aus den Reticuloendothelien entstehen können.

Auch funktionell sind diese Reticulumzellen in nichts von den übrigen Reticulo-

endothelien verschieden. Gerade in den FLEMMING'schen und Übergangssekundärknötchen kann man oft eine starke Phagocytose beobachten, wie häufig genug beschrieben und abgebildet worden ist. Auch die Gitterfaserbildung ist nicht schwer bei ihnen nachzuweisen. In den Übergangssekundärknötchen, welche, wie gesagt, sehr reich an diesen Zellen sind, können sie sehr reichliche und kräftige Fasern bilden (s. Abb. 9). Daß die FLEMMING'schen und Übergangssekundärknötchen im allgemeinen nicht so reich an Gitterfasern sind als das sie umgebende lymphoide Gewebe, ist hauptsächlich Ausdruck ihres geringen Alters. Je jünger sie sind, um so weniger und dünnere Gitterfasern werden wir in ihnen erwarten.

Daß diese Zellen echte ausdifferenzierte Reticuloendothelien sind und sich nicht mehr in Lymphocyten umbilden können, erhält eine weitere Stütze durch die Beobachtung, daß man gerade im Anfang unserer Experimente, die wohl alle mit regressiven Veränderungen in den Sekundärknötchen losgingen, häufig feststellen konnte, daß die in Rede stehenden Reticulumzellen sich mit Kerntrümmern vollbeladen hatten, während zu gleicher Zeit eine Neubildung von großen Lymphocyten stattfand. Dabei entstanden die großen Lymphocyten hier genau so, wie überall anderswo im lymphoiden Gewebe, von den undifferenzierten Mesenchymzellen. Auch hier entstanden sie besonders entlang den Gefäßen, wodurch häufig jene strangförmige Anordnung zustande kam, wie sie von MAXIMOW (1927) beschrieben worden ist.

Betrachten wir nun noch einmal die Streitpunkte in der Lymphopoëse, so sehen wir, daß jene Autoren, die wie ASCHOFF und seine Schüler eine Entstehung von Lymphocyten aus den Reticuloendothelien leugnen, völlig recht haben. Diese Zellen sind bereits mehr oder weniger ausdifferenziert und können keine Lymphocyten mehr bilden. Aber auch jene Autoren, welche wie MAXIMOW und seine Schüler eine Entstehung der Lymphocyten aus fixen Zellen annehmen, haben Recht. Die eigentlichen Mutterzellen der Lymphocyten sind auch im postfetalen Leben die fixen undifferenzierten Mesenchymzellen, aus denen sie genau so entstehen, wie von ALFEJEW (1924) für das Fetalleben beschrieben. Eine weitere Vermehrung der Lymphocyten findet dann durch Mitose statt, wie durch ihre häufigen Kernteilungsfiguren bewiesen ist. Jene Ansicht aber, welche die großen Lymphocyten durch Hypertrophie aus kleinen hervorgehen läßt, und diese großen Lymphocyten dann als Mutterzellen für neue Lymphocyten ansieht, ist unbegründet und widerspricht auch den bekannten Befunden bei der Entstehung der polymorphkernigen Leukocyten.

Wir können somit auch bei der Lymphopoëse 2 Modi unterscheiden,

einen homeoplastischen und einen heteroplastischen. Welchem von beiden Prozessen die größere Bedeutung zuzumessen ist, ist schwer zu entscheiden und hängt wohl von der Stärke der Reizung und der Anzahl der großen Lymphocyten ab, die zur Zeit des Lymphocytenbedarfs vorhanden sind. Unter physiologischen Bedingungen, wo der Verbrauch von Lymphocyten nur relativ gering ist, mag die Vermehrung durch Mitose ausreichen, den Lymphocytenbedarf zu decken. Bei einigermaßen erhöhten Anforderungen genügt aber dieser Modus nicht. Hier werden dann große Lymphocyten aus den undifferenzierten Mesenchymzellen neugebildet.

Die Umwandlung der großen in mittelgroße und kleine Lymphocyten findet wahrscheinlich nicht durch Mitose statt, wenn auch ein solcher Vorgang vielleicht im Sinne einer pathologischen Teilung vorkommen mag. Im allgemeinen müssen wir wohl annehmen, daß das Kleinerwerden dieser Zellen durch Eindickung oder Schrumpfung erfolgt, wofür neben HEIBERG's Messungen an den Äquatorialplatten (1924) die Tatsache spricht, daß der Chromatingehalt der Kerne mit Abnahme der Größe der Zellen immer stärker wird.

2. *Die Lymphocytenemigration*

Die Lymphocyten bewegten sich meist schnell von dem Orte ihrer Entstehung fort und drangen überall in das lymphatische Gewebe und in die Sinus ein. Dabei beteiligten sich auch die großen Lymphocyten, wie oft zu beobachten war. Bei starker Vermehrung häuften sich die großen Lymphocyten oft in dicken Mänteln um die Gefäße an, wie besonders im 1. Experiment mit Staphylokokkenvaccine deutlich war. Hier vermehrten sich die großen Lymphocyten anscheinend schneller, als ihre Abwanderung und Umwandlung in kleine Lymphocyten erfolgen konnte.

Die Lymphocyten wandern, wie ich schon früher betont habe (1929), auf 2 Wegen ins Blut, teils durch die Sinus und durch den Ductus thoracicus, und teils durch die postkapillären Venen des lymphoiden Gewebes. Während HELLY (1914), SCHRIDDE (1921) und ASCHOFF (1926) der Ansicht waren, daß die Lymphocyten in den Kapillaren der Sekundärknötchen in die Blutbahn überträten, habe ich in Übereinstimmung mit SCHUMACHER (1899) und MAXIMOW (1927) gezeigt, daß die Auswanderung in den postkapillären Venen stattfindet, in

deren Wand und Lumen man oft zahlreiche Lymphocyten sehen kann. Daß hier Lymphocyten ins Blut übertreten, hat schon SCHUMACHER erwiesen, der die Leukocytenzahlen der zu- und abführenden Blutgefäße von Lymphknoten verglichen hat. Ich habe (1929 a) in Abb. 12 eine Arterie und Vene zusammen im gleichen Gesichtsfeld abgebildet, die hieran keinen Zweifel lassen.

Bei unseren 4 Experimenten fand die stärkste Lymphocytenemigration durch Sinus und Venen dann statt, wenn die großen Lymphocyten an Zahl bereits wieder abnahmen (Tabelle 1) und sich in kleine Lymphocyten umwandelten. Es war in jener Zeit, wo die Hyperplasie ihren Höhepunkt erreichte und kurz hinterher. Die stärkste Lymphocytenemigration ging mit der stärksten Vermehrung der Blutlymphocyten parallel, die ihren Gipfel kurz nach der kräftigsten Lymphocytenemigration erreichten.

TABELLE 1

Tab. 1. Zusammenfassung einiger Ergebnisse der 4 Experimente. In der Tabelle sind die Versuchstage zusammengestellt, an welchen die Eiterung an der Injektionsstelle ihre größte Ausdehnung hatte, die reticuloendotheliale Reaktion und die lymphatische Hyperplasie am stärksten entwickelt und die Zahl der großen Lymphocyten im lymphatischen Gewebe, die Lymphocytenemigration, die Blutlymphocytenzahl und die Zahl und Größe den FLEMMING'schen Sekundärknötchen oder der sog. Keimzentren auf ihrem Höhepunkt waren.

Injiziertes Material	Eiterung	Reticulo-endo-theliale Reaktion	Große Lympho-cyten	Lympha-tische Hyper-plasie	Lympho-cyten-emi-gration	Blut-lympho-cyten	„Keim-zentren“
Arsenige Säure	—	1–3	2–3	3–6	3–7	10	3–21
Staphylokokkenvaccine	4	2–4	3–4	5–6	4–7	9	5–28
Benzol	5–8	2–8 (14)	4–6	6–7	6–12	14	6–28
Terpentinöl	5–8	2–8 (14)	4–6	8–10	8–13	13	8–27

Wenn wir auch eine deutlich vermehrte Lymphocytenemigration durch die Venen beobachten konnten, so sahen wir im allgemeinen doch nicht so starke Grade, wie ich sie früher beobachtet und abgebildet hatte (1929 c). Dort war es zu einer so starken Hyperplasie gekommen, daß die Sinus fast überall zusammengedrückt und kaum noch zu erkennen waren. Daraus geht hervor, daß, je stärker die Hyperplasie

ist und je mehr die Sinus durch das wachsende Gewebe komprimiert werden, desto mehr Lymphocyten durch die Venen ins Blut gelangen.

3. Die Entstehung der Sekundärknötchen und ihr Schicksal

Wie ich schon zu Anfang des 2. Abschnitts ausgeführt habe, sind die echten soliden Sekundärknötchen immer primäre Bildungen. Sie sind entwicklungsgeschichtlich die ersten und werden auch im postfetalen Leben stets primär im lymphoiden Gewebe gebildet. Auch in den hier mitgeteilten Versuchen waren sie die ersten Sekundärknötchen, welche neugebildet wurden. Sie bildeten sich am Rande der Rinde um kleine Arterien und Kapillaren, wie an geeigneten Schnitten zu ersehen war, indem sich große Lymphocyten aus den undifferenzierten Mesenchymzellen ablösten und sich schnell zu kleinen Lymphocyten weiterentwickelten. Daß sie hier nicht aus den normalerweise vorhandenen Übergangs- oder FLEMMING'schen Sekundärknötchen entstanden waren, geht daraus hervor, daß diese zu dieser Zeit regressive Veränderungen aufwiesen und viel reicher an Reticulumzellen und meist auch Gitterfasern waren als die soliden Sekundärknötchen. Auch waren die soliden Sekundärknötchen zunächst viel kleiner als die anderen.

Für die Ansicht von GROLL und KRAMPF (1920/1) und WAETJEN (1925), daß die soliden Sekundärknötchen aus FLEMMING'schen hervorgehen und gewissermaßen ihre Ruhephase sind, habe ich auch hier keine Anhaltspunkte finden können. Bei meinen mit kleinen Arsendosen ausgeführten Versuchen fand ich zwar ähnlich wie WAETJEN einige Tage nach der Injektion vorwiegend solide Sekundärknötchen in den regionären Lymphknoten. Bei der Untersuchung dieser Tiere in Zeitserien fand sich jedoch, daß es in den ersten Stunden nach der Injektion zu einem hochgradigen Zerfall der Sekundärknötchen gekommen war, dem dann eine Neubildung von soliden Sekundärknötchen folgte. Es soll nicht geleugnet werden, daß man durch sehr kleine Dosen Arsen evtl. eine direkte Umwandlung von den großen und mittelgroßen Lymphocyten der FLEMMING'schen Sekundärknötchen in kleine Lymphocyten hervorrufen kann. Wenn das aber überhaupt möglich ist, sind dazu viel kleinere Dosen nötig, als WAETJEN und ich sie benutzt haben. Diese Umwandlung würde auch nicht in der Neubildung von soliden, sondern von Pseudosekundärknötchen resultieren, wie aus dem Folgenden hervorgeht.

Die neugebildeten soliden Sekundärknötchen wuchsen eine Zeitlang und zwar so lange, als sich reichlich große Lymphocyten in ihnen fanden. Ihr Schicksal war, zu Pseudosekundärknötchen weiterzu-

wachsen, wie sich durch Übergangsbilder belegen ließ, oder durch Entwicklung eines hellen Zentrums zu FLEMMING'schen Sekundärknötchen zu werden. Andere lösten sich mehr diffus im lymphoiden Gewebe auf.

Die ersten FLEMMING'schen Sekundärknötchen oder Keimzentren entwickelten sich vom 3.–8. Tage unserer Experimente an, d. h. erst dann, wenn die lymphatische Hyperplasie ihrem Höhepunkt nahe war (Tabelle 1). Mit voller Ausbildung dieser Sekundärknötchen hatte auch die Hyperplasie ihren Höhepunkt erreicht. Dabei waren auch sie vielfach primäre Bildungen im lymphoiden Gewebe, und zwar dann, wenn sie keine Randzone oder Kappe besaßen. Man sah solche Sekundärknötchen besonders nach stärkeren Reizen, wo sehr viele Sekundärknötchen gebildet wurden, und zwar besonders im Innern von Mark und Rinde. Bei schwachen Reizen traten sie jedoch häufig nur sekundär in den soliden Sekundärknötchen auf, wie schon von GROLL und KRAMPF (1920/1), HEIBERG (1923) und POL (1923) beobachtet. Sie fanden sich dann vorwiegend nur am Rande der Rinde.

Die FLEMMING'schen Sekundärknötchen entwickelten sich ähnlich wie die soliden im Anschluß an Arteriolen und Kapillaren, indem sich große Lymphocyten von den sie begleitenden undifferenzierten Mesenchymzellen loslösten. Der Unterschied gegen die Bildung von soliden Sekundärknötchen bestand nur darin, daß sich bei diesen die großen Lymphocyten sehr schnell in kleine umwandelten, während sie sich bei den FLEMMING'schen Sekundärknötchen nur zu mittelgroßen Lymphocyten umbildeten, womit ihre Entwicklung einstweilen abgeschlossen war. Die großen und mittelgroßen Lymphocyten vermehrten sich weiter durch Mitose, bis schließlich große, wohl geformte, scharf abgegrenzte helle Zentren bis zu 0,75 mm Durchmesser ausgebildet waren.

Bei der Entstehung der FLEMMING'schen Sekundärknötchen aus den soliden entstand das helle Zentrum teils in der Mitte der soliden Sekundärknötchen, so daß es überall von einer mehr oder weniger gleichmäßig dicken Randzone umgeben war (Abb. 46 a–c). Je größer es wurde, um so dünner wurde die Randzone. In anderen Knötchen entstand das helle Zentrum mehr der Eintrittsstelle der Arteriole zu, woraus beim Weiterwachsen eine Kappenbildung

resultierte (Abb. 46 a, d und e). Schließlich konnte man nicht selten jene schon von HEIBERG (1923) beschriebenen Bilder sehen, wo sich der distale, dem Randsinus zugekehrte und der Eintrittsstelle der Arteriole abgekehrte Teil des hellen Zentrums bereits regressiv umgewandelt hatte, während der proximale Teil noch weiterwuchs (Abb. 46 f). 1929 a (Abb. 14) habe ich sogar ein Sekundärknötchen abgebildet, wo sich neben einem Übergangssekundärknötchen an der Eintrittsstelle der Arteriole ein helles Zentrum neugebildet hatte (Abb. 46 g).

Aus den hier mitgeteilten Beobachtungen geht zur Genüge hervor, einen wie großen Einfluß der Ort der Entstehung des hellen Zentrums auf die Form der FLEMMING'schen Sekundärknötchen und der Rand-

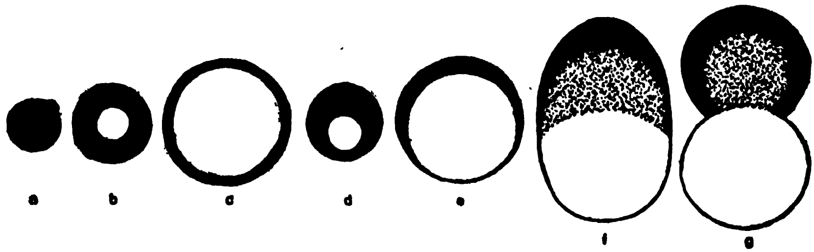

ABB. 46. Schema der Entstehung von FLEMMING'schen Sekundärknötchen mit Randzone und Kappe: a = solides Sekundärknötchen, b–c = Entstehung einer Randzone, d–e = Entstehung einer Kappe, f = distaler Zerfall und proximales Weiterwachsen eines hellen Zentrums, g = Neuentstehung eines hellen Zentrums proximal von einem Übergangssekundärknötchen.

zone hat. Er ist in der Tat der ausschlaggebende Faktor. Sekundär spielen dann die von RÖHLICH (1928) eingehend untersuchten Spannungen innerhalb des Gitterfasersystems eine Rolle und, was die Kappenbildung betrifft, auch die Umwandlung der großen und mittelgroßen Lymphocyten in kleine, worauf SCHWANEN (1929 a) so großen Wert gelegt hat. Auf den letzten Punkt werde ich noch zurückkommen.

Die Übergangssekundärknötchen sind, wie ich schon früher (1929 a) in Übereinstimmung mit HELLMAN (1918/9, 1921) und HEIBERG (1923) ausgeführt habe, meist nichts weiter als Auflösungsstadien der FLEMMING'schen Sekundärknötchen oder zur Ruhe gekommene FLEMMING-sche Sekundärknötchen, wie MAXIMOW (1927) sie nennt. Mit der Rückbildung der FLEMMING'schen Sekundärknötchen gehen, wie von HUECK (1927), SCHWANEN (1929 b) und JÄGER (1929) gezeigt wurde,

oft regressive Veränderungen an den sie versorgenden Gefäßen einher. Ob man dabei die regressiven Gefäßveränderungen als Ursache der Rückbildung der FLEMMING'schen Sekundärknötchen auffassen darf, scheint mir zweifelhaft. Es kann zwar keinem Zweifel unterliegen, daß stärkere Gefäßveränderungen zur Rückbildung führen. Man kann das oft genug in Milzen mit Arteriolosklerose beobachten. Solche Veränderungen fehlen aber in den Übergangssekundärknötchen für gewöhnlich. Gegen eine solche Auffassung spricht auch die Beobachtung, daß die Übergangssekundärknötchen jederzeit wieder zu wachsen anfangen können.

Während die Übergangssekundärknötchen für gewöhnlich nicht weiterwachsen, sondern sich mehr und mehr im lymphoiden Gewebe auflösen, können sie, wenn die Lymphknoten von einem neuen Reiz getroffen werden, auch wieder neu zu wachsen anfangen. Wir haben solche Bilder häufig zu Beginn unserer Versuche gesehen. Wir sahen dabei in den Übergangssekundärknötchen neue und vermehrte große Lymphocyten auftreten, die meist von den undifferenzierten Mesenchymzellen ihren Ursprung nahmen. Sie bildeten sich zu mittelgroßen und kleinen Lymphocyten weiter. Nirgends habe ich aber gesehen, daß sie zu einem neuen FLEMMING'schen Sekundärknötchen wurden, d. h. daß in ihnen ein neues, wohl begrenztes, nur aus großen und mittelgroßen Lymphocyten bestehendes helles Zentrum entstand, wie es MAXIMOW (1927) für die Regel hält. Diese Sekundärknötchen wuchsen vielmehr unter ständiger Bildung von kleinen Lymphocyten weiter, bis sie schließlich zu Pseudosekundärknötchen wurden oder sich diffus im lymphoiden Gewebe auflösten.

Die Pseudosekundärknötchen gehen, wie ich schon im Anfang dieses Abschnitts beschrieben habe, im Fetalleben ausschließlich aus den soliden Sekundärknötchen hervor. Da sich nun auch im postfetalen Leben alle Übergänge zwischen beiden nachweisen lassen, muß man auch hier eine solche Entstehung zulassen.

In den hier mitgeteilten Experimenten haben wir nun auch Bilder gesehen (Abb. 34), die keine andere Deutung als die einer direkten Umwandlung von FLEMMING'schen Sekundärknötchen in Pseudosekundärknötchen erlauben. Wir sahen, wie sich die großen und mittelgroßen Lymphocyten plötzlich in kleine Lymphocyten umwandelten. Teilweise Umwandlungen dieser Art habe ich in Übereinstimmung mit

SCHWANEN (1929 a) häufiger dort gesehen, wo das helle Zentrum dem Randsinus oder Epithel am nächsten lag. Komplette Umwandlungen von ganzen hellen Zentren in kleine Lymphocyten sind aber nur sehr selten zu beobachten und fanden sich in unseren Experimenten nur in jener Zeit, wo die ersten FLEMMING'schen Sekundärknötchen in Erscheinung traten, d. h. zu einer Zeit, wo sich die Tendenz der Lymphknoten, kleine Lymphocyten zu produzieren, mit der Tendenz, FLEMMING'sche Sekundärknötchen zu bilden, überschnitt. Jedenfalls geht hieraus mit Sicherheit hervor, daß eine direkte Umwandlung von FLEMMING'schen zu Pseudosekundärknötchen vorkommt. Daß hierbei nicht echte solide Sekundärknötchen resultieren, geht einerseits aus der histologischen Struktur dieser Gebilde und andererseits aus ihrer Größe hervor.

Eine dritte und letzte Quelle für die Pseudosekundärknötchen sind schließlich die Übergangssekundärknötchen. Übergänge von ihnen zu Pseudosekundärknötchen sind keine Seltenheit. Wie wir in unseren Experimenten beobachten konnten, entstehen sie teils dadurch, daß ein bereits zur Ruhe gekommenes Übergangssekundärknötchen, das keine großen Lymphocyten mehr enthält, wieder neu zu wachsen anfängt, was wir besonders im Anfang unserer Experimente beobachtet haben. Ein andermal entstehen sie dadurch, daß sich ein FLEMMING'sches Sekundärknötchen mehr und mehr in ein Übergangssekundärknötchen umwandelt, wobei ein Teil der mittelgroßen Lymphocyten zerfällt oder sich in kleine Lymphocyten verwandelt (besonders am distalen Teil der hellen Zentren), und ein anderer Teil noch weiterwächst, indem sich neue große Lymphocyten aus dem undifferenzierten Mesenchym herauslösen (besonders am proximalen Teil der hellen Zentren). Schließlich werden sie den Pseudosekundärknötchen immer ähnlicher und kann man sie nicht mehr von ihnen unterscheiden. Diesen letzten Modus haben wir besonders im weiteren Verlauf unserer Experimente beobachtet.

Die Pseudosekundärknötchen verschwinden schließlich dadurch, daß die Lymphocyten teils abwandern; teils aber auch dadurch, daß die Pseudosekundärknötchen neue Sinus erhalten, bis sie wieder wie diffuses lymphoides Gewebe aussehen.

In Abb. 47 habe ich die Beziehungen der Sekundärknötchen zueinander und zum lymphoiden Gewebe noch einmal kurz zusammen-

gestellt. Was hierbei die häufigsten Modi sind, ist schwer zu entscheiden. Im Fetalleben sind jedenfalls a–c, a–c–e und a–e die einzigen. Im postfetalen Leben kommen die FLEMMING'schen Sekundärknötchen oder die sog. Keimzentren dazu. Sie entstehen wohl meist, wenigstens bei schwachen, physiologischen Reizen, durch Entwicklung eines hellen Zentrums in den soliden Sekundärknötchen. Bei stärkeren, pathologischen Reizen treten sie auch primär im lymphoiden Gewebe auf. Sie verschwinden meist auf dem Wege

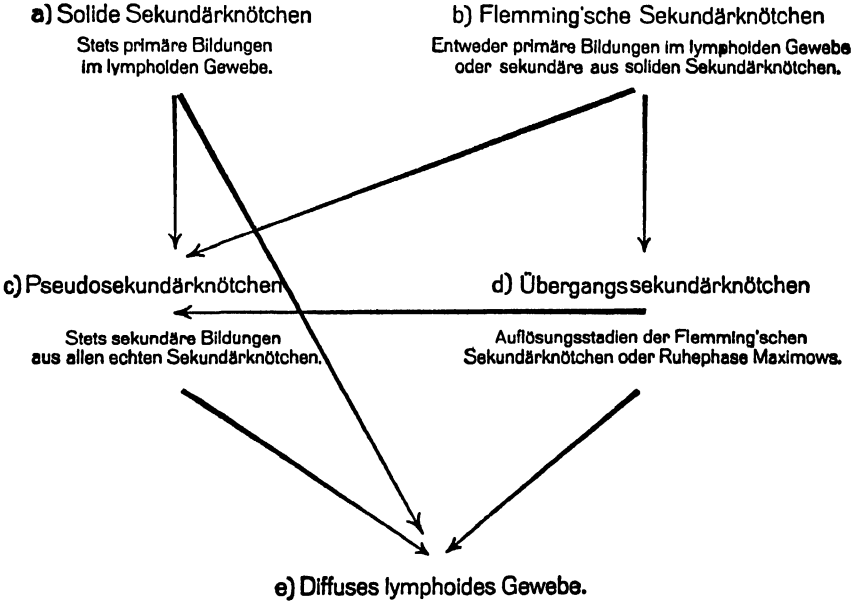

ABB. 47. Die Entstehung der Sekundärknötchen und ihre Entwicklungsmöglichkeiten.

b–d–e. Die Pseudosekundärknötchen können auch im postfetalen Leben auf dem Wege a–c entstehen. Wenn FLEMMING'sche Sekundärknötchen vorhanden sind, bilden sie sich vielfach aus diesen auf dem Umwege über Übergangssekundärknötchen (b–d–c), indem entweder der proximale Teil des hellen Zentrums weiterwächst, während sich der distale Teil schon auflöst, oder indem ein schon zur Ruhe gekommenes Übergangssekundärknötchen wieder neu zu wachsen anfängt. Es kommt auch eine direkte Umwandlung von

FLEMMING'schen zu Pseudosekundärknötchen vor (b–c). Sie scheint aber sehr selten zu sein.

4. *Die Bedeutung der Sekundärknötchen und des lymphoiden Gewebes*

GROLL und KRAMPF (1920/21) hatten die Theorie aufgestellt, daß die soliden Sekundärknötchen zur Ruhe gekommene FLEMMING'sche Sekundärknötchen wären, und WAETJEN (1925) glaubte, dieser Theorie eine experimentelle Stütze gegeben zu haben. Die Nachprüfung der Untersuchungen von WAETJEN ergab jedoch, daß seine Befunde anders gedeutet werden müssen und es sich dabei nicht um eine Umwandlung von FLEMMING'schen Sekundärknötchen oder sog. Keimzentren, sondern vielmehr um eine Neubildung von soliden Sekundärknötchen gehandelt hatte. Wie ich im vorigen Paragraphen gezeigt habe, sind die soliden Sekundärknötchen stets primäre Bildungen im lymphoiden Gewebe. Sie sind überhaupt die ersten Sekundärknötchen, welche gebildet werden. Sie treten sehr früh im Embryonalleben in Erscheinung, zu einer Zeit, wo es noch gar keine FLEMMING'schen Sekundärknötchen gibt. Sie treten ferner auch im postfetalen Leben stets primär auf. Aus diesen Gründen können wir die soliden Sekundärknötchen nicht als Ruhephase der FLEMMING'schen Sekundärknötchen auffassen, sondern müssen ihnen eine eigene Bedeutung zuerkennen.

Die soliden Sekundärknötchen sind nun, wie ich oben gezeigt habe, knötchenförmige Anhäufungen von kleinen Lymphocyten, die um Arteriolen und Kapillaren aus den undifferenzierten Mesenchymzellen über große und mittelgroße Lymphocyten entstanden sind. Im ruhenden Zustand fehlen große und mittelgroße Lymphocyten so gut wie ganz. Im wachsenden Zustand können sie aber reichlich solche Zellen und auch Kernteilungsfiguren enthalten. Daß man in ihnen niemals so viele große und mittelgroße Lymphocyten sieht, wie in den FLEMMING'schen Sekundärknötchen, ist nur ein Ausdruck dafür, daß ihre Umwandlung in kleine Lymphocyten sehr schnell vor sich geht. Daß es sich hier in der Tat um eine schnelle Umwandlung handelt, wird besonders dadurch gezeigt, daß sich die soliden Sekundärknötchen immer besonders dann finden, wenn ein großer Bedarf an kleinen Lymphocyten vorliegt. Wir sehen sie nämlich im Embryonalleben und in den ersten postfetalen Monaten, wo ein starkes Wachstum

aller lymphatischen Organe stattfindet und auch das Blut besonders viele kleine Lymphocyten enthält. Wir fanden sie auch im Anfang aller unserer Experimente, als das lymphatische Gewebe am stärksten wuchs. Aus diesen Befunden müssen wir den Schluß ziehen, daß die soliden Sekundärknötchen Ausdruck lokalisierter, besonders lebhafter Lymphocytenvermehrung sind, die mit der Bildung von kleinen Lymphocyten einhergeht.

Daß nun auch die FLEMMING'schen Sekundärknötchen Orte lebhafter Lymphocytenvermehrung sind, kann auf Grund ihrer zahlreichen Mitosen gar nicht bezweifelt werden und ist auch von fast niemandem ernstlich in Frage gestellt worden. Hingegen ist FLEMMING's Theorie angezweifelt worden, nach welcher die großen und mittelgroßen Lymphocyten der hellen Zentren ständig kleine Lymphocyten liefern sollen, die dann durch die Randzone ins umliegende Gewebe auswanderten. Wie von den Gegnern dieser Theorie immer wieder ins Feld geführt wird, stützt sich FLEMMING's Theorie eigentlich nur auf die Anwesenheit der oft zahlreichen Mitosen. Auch MAXIMOW (1927), der erst kürzlich wieder für diese Theorie eingetreten ist, konnte keine neuen Beweise für sie herbeibringen.

Jene Autoren, welche wie WAETJEN (1925, 1929), ASCHOFF (1926) und HUECK (1928) angeblich einen vermittelnden Standpunkt eingenommen haben, sind in Wirklichkeit Anhänger der FLEMMING'schen Theorie. Ihre Auffassung läßt sich am besten in der Theorie ASCHOFF's zusammenfassen, nach welcher jene „Keimzentren", welche reich an großen und mittelgroßen Lymphocyten und Kernteilungsfiguren sind, kleine Lymphocyten im Sinne FLEMMING's bilden sollen, während solche, welche reich an Reticuloendothelien sind, Abwehrherde darstellen. Bei der Kontroverse stehen aber nur die ersteren, die eigentlichen FLEMMING'schen Sekundärknötchen zur Diskussion.

Gegen die FLEMMING'sche Theorie sind nun gewichtige Gründe erhoben worden. MARCHAND (1913) war der erste, welcher Zweifel erhob, indem er auf die oft scharfe Abgrenzung der hellen Zentren hinwies, dic einen Übergang der mittelgroßen Lymphocyten der hellen Zentren in die kleinen Lymphocyten der Randzone oder des lymphoiden Gewebes vermissen lasse. Diese Beobachtung ist immer wieder bestätigt worden. Selbst Autoren wie SCHWANEN (1919 a), welcher an der FLEMMING'schen Theorie festhalten, geben zu, daß „die fehlenden Übergänge von Sekundärknötchenzellen in fertige Lymphocyten

nach wie vor eine Lücke in der Erklärung bilden". Er hat deshalb angenommen, daß die Sekundärknötchen durch dauernde Reize geschädigt seien, was sich darin äußere, daß trotz größter Anstrengung und des ausgedehntesten Wachstums in ihnen nicht genügend Lymphocyten produziert werden könnten, eine Erklärung, die wohl kaum mit Ernst herangezogen werden kann.

Die wichtigsten Einwände sind dann von HELLMAN (1913/4, 1918/9, 1921, 1926) erhoben und in der Folgezeit zum größten Teil bestätigt worden. Er wies auf das quantitative Mißverhältnis zwischen hellen Zentren und Randzonen hin, von denen man erwarten sollte, daß sie sich mit der Größe des Zentrums und der Zahl der Mitosen verbreitern würden. Dieses ließ sich aber nicht zeigen. Man sieht im Gegenteil sogar häufig sehr große helle Zentren mit zahlreichen Mitosen ohne alle Randzonen. Besonderes Gewicht legte er aber auf die Beobachtung, daß gerade dann, wenn sehr viele Lymphocyten produziert würden, die hellen Zentren oft ganz fehlten (z. B. bei lymphatischer Leukämie), und daß sich auf der anderen Seite bei vielen Infektionskrankheiten und beim Status thymico-lymphaticus oft geradezu massenhaft helle Zentren fänden, ohne daß gleichzeitig eine Lymphocytenvermehrung im Blut bestände. Hierauf hatte auch schon NÄGELI (1923) hingewiesen. Eine ähnliche Diskrepanz fand er auch in der Altersentwicklung des lymphatischen Gewebes und der Blutlymphocytenzahl.

HEIBERG (1923, 1924, 1924/5, 1927) hat weiter hervorgehoben, daß die meisten FLEMMING'schen Sekundärknötchen schon Anzeichen einer Auflösung zeigten, und daß viele von ihnen reichlich Kerntrümmer und Phagocyten enthielten.

Alle diese Befunde führten die bisher genannten Autoren zur Ablehnung der FLEMMING'schen Theorie. Ihnen haben sich weiter DIETRICH (1923), SCHLEMMER (1923), FÖRSTER (1923), POL (1923), BERNHEIM (1924), HEILMANN (1925, 1926, 1927), WETZEL (1926), CATANIA (1927), PETERSEN (1930), SJOEVALL und SJOEVALL (1930) u. A. angeschlossen.

Ich habe die bisher genannten Argumente gegen FLEMMING'S Theorie um einige vermehren können (1929 a und c). Wegen der fehlenden Übergänge der großen und mittelgroßen Lymphocyten der hellen Zentren zu den kleinen der Randzonen hatten einige Autoren

angenommen, daß die kleinen Lymphocyten, welche im Zentrum gebildet würden, in ihnen durch die Kapillaren ins Blut abwanderten. Ich habe aber gezeigt, daß die Lymphocytenemigration in die Blutgefäße außerhalb der echten Sekundärknötchen durch besonders gebaute Venen erfolgt, welche vor allem in den Pseudosekundärknötchen reichlich sind. Ferner habe ich HELLMAN's Beobachtung über die Diskrepanz zwischen FLEMMING'schen Sekundärknötchen und Blutlymphocyten im Experiment bestätigen können. Bei starker Reizung mit lebenden Staphylokokken war es zunächst zu einer den ganzen Lymphknoten ausfüllenden, diffusen lymphoiden Hyperplasie gekommen, die mit einer Lymphocytenvermehrung im Blut verbunden war. Erst als die Zahl der Blutlymphocyten bereits wieder abnahm, bildeten sich FLEMMING'sche Sekundärknötchen.

Auch die in dieser Arbeit mitgeteilten Versuche, denen viel schwächere Reize zugrunde lagen als den eben erwähnten, lassen sich mit der FLEMMING'schen Theorie nicht in Einklang bringen und bestätigen wieder die Diskrepanz von der Bildung FLEMMING'scher Sekundärknötchen und der Blutlymphocyten. Die FLEMING'schen Sekundärknötchen traten erst dann auf, wenn die Lymphocytenemigration bereits ihren Höhepunkt erreicht hatte und blieben in allen 4 Versuchen in voller Ausdehnung bis ans Ende der Versuche erhalten, d. h. bis 14–21 Tage nach der Lymphocytenemigration aus den Lymphknoten oder bis 11–14 Tage nach den Gipfeln der Lymphocytenvermehrung im Blut (Tabelle 1). Sollte die FLEMMING'sche Theorie zu Recht bestehen, hätte die größte Lymphocytenauswanderung und die stärkste Vermehrung der Blutlymphocyten 14 Tage später stattfinden müssen. Der Anteil der FLEMMING'schen Sekundärknötchen an der Lymphocytenemigration schien vielmehr ein rein mechanischer zu sein, indem die wachsenden Knötchen die Auswanderung der umherliegenden Lymphocyten durch Verdrängung förderte.

Alle diese Beobachtungen sprechen entschieden gegen FLEMMING's Theorie, lassen sich aber, wie ich schon früher gesagt habe (1929 a), mit ihr in Einklang bringen, wenn wir sie dahin modifizieren, daß wir sie nicht als ständig kleine Lymphocyten bildende Orte, sondern gewissermaßen als Reservedepots an großen und mittelgroßen Lymphocyten auffassen, die hier im Sinne einer Regeneration im Überschuß gebildet wurden. Gegen diese Theorie schienen mir damals zwei Punkte zu

sprechen: 1. daß ich keine direkten Umwandlungen der hellen Zentren in Pseudosekundärknötchen, d. h. keine nennenswerte Umwandlung der mittelgroßen Lymphocyten dieser Zentren in kleine gesehen hatte, und 2. daß man im Gegensatz zu den vielen FLEMMING'schen Sekundärknötchen immer nur sehr spärlich Pseudosekundärknötchen findet, und bei Richtigkeit der Reservedepottheorie doch Bilder hätte sehen sollen, wo sich alle oder nahezu alle FLEMMING'schen Sekundärknötchen in solche umgewandelt hatten.

Die erste Schwierigkeit ist damit behoben worden, daß ich in dieser Arbeit direkte Umwandlungen von FLEMMING'schen in Pseudosekundärknötchen nachweisen konnte. Ob die zweite Schwierigkeit nun allein durch im Überschuß stattfindende Regeneration erklärt werden kann, ist besonders deswegen schwer zu entscheiden, weil die Diskrepanz zwischen der Zahl der FLEMMING'schen und Pseudosekundärknötchen oft sehr groß ist.

Die hellen Zentren sind nun, wie ich verschiedentlich betont habe, Orte, in denen die Lymphocytenbildung nicht, wie in den soliden Sekundärknötchen, rasch zu kleinen Lymphocyten führt, sondern in denen die Lymphopoëse mit der Ausbildung von mittelgroßen Lymphocyten stehen bleibt. Die hellen Zentren bleiben eine ganze Weile bestehen, ohne daß sich kleine Lymphocyten bilden, wobei die mittelgroßen Lymphocyten z. T. zerfallen, wie durch die oft reichlichen tingiblen Körperchen und alle Übergänge zu ihnen nachgewiesen ist, und wobei sich sogar immer neue mittelgroße Lymphocyten durch Mitose bilden. Ihr endgültiges Geschick ist dann gewöhnlich regressive Umwandlung.

Diese Beobachtungen und Überlegungen lassen daran denken, daß hier besondere mittelgroße Lymphocyten mit einer besonderen Funktion vorliegen. Besonders ASCHOFF (1926) hat darauf hingewiesen, daß den verschiedenen Lymphocyten in den verschiedenen Teilen des lymphatischen Gewebes möglicherweise ganz verschiedene Funktionen obliegen. Die mittelgroßen Lymphocyten der FLEMMING'schen Sekundärknötchen sollen vielleicht für gewöhnlich gar keine kleinen Lymphocyten bilden und haben vielleicht nur ganz nebenbei die Fähigkeit, sich in kleine Lymphocyten zu verwandeln, wenn ein neuer, kleine Lymphocyten bildender Reiz die FLEMMING'schen Sekundärknötchen trifft. Wir haben direkte Umwandlungen

von FLEMMING'schen Sekundärknötchen in kleine Lymphocyten bei unseren Versuchen ja auch nur dann beobachtet, wenn die ersten Sekundärknötchen auftraten, und die kleine Lymphocyten bildenden Reize zwar im Erlöschen, aber noch vorhanden waren.

Was die eigentliche Funktion der FLEMMING'schen Sekundärknötchen sein könnte, wenn sie nicht nur Reservedepots wären, ist völlig der Spekulation überlassen. Da HELLMAN sie immer in Abhängigkeit von Infektionen auftreten sah, hat er sie als Reaktionszentren bezeichnet, eine Bezeichnung, die durchaus das Wesen dieser Knötchen trifft, was sie auch sein mögen. Damit überein stimmt auch der Befund, daß wir sie normalerweise nur in den von den Schleimhäuten des Verdauungstraktes abhängigen lymphatischen Geweben finden, während sie in der Milz meist fehlen und in den peripheren Lymphknoten nur selten vorhanden sind. Ob man sie als Resorptionszentren ansprechen kann, wie DIETRICH getan hat, ist unsicher. Bevor wir Weiteres über sie aussagen können, müssen wir wohl erst mehr über die Funktion der Lymphocyten wissen.

Die Übergangssekundärknötchen sind für gewöhnlich zur Ruhe gekommene FLEMMING'sche Sekundärknötchen, oder mit anderen Worten Auflösungsstadien derselben. Bei Lymphocytenbedarf können sie wieder zu wachsen anfangen und neue kleine Lymphocyten bilden, während sie, wie oben beschrieben, nicht wieder zu FLEMMING'schen Sekundärknötchen werden könne. Die Übergangssekundärknötchen haben also keine eigentliche Funktion. Ihre Bestimmung ist, im lymphoiden Gewebe aufzugehen.

Die Deutung der Pseudosekundärknötchen dürfte keinen Meinungsverschiedenheiten begegnen. Sie sind das Resultat stattgehabter Bildung kleiner Lymphocyten, sei es, daß sich solide Sekundärknötchen immer mehr vergrößert hatten, sei es, daß Übergangssekundärknötchen von neuem gewachsen waren, oder sei es, daß sich FLEMMING'sche Sekundärknötchen direkt in Pseudosekundärknötchen umgewandelt hatten. Solange sie noch große und mittelgroße Lymphocyten enthalten, produzieren sie auch noch kleine Lymphocyten, deren Schicksal es ist, zu Blutlymphocyten zu werden. Die Lymphocyten wandern teils durch die Sinus und teils durch die in den Pseudosekundärknötchen vorhandenen Venen ins Blut aus. Sie finden sich oft in großen Mengen in der Wand und im Lumen dieser Venen.

Die Pseudosekundärknötchen sind somit Ansammlungen von blutreifen Lymphocyten, die mit allen Vorrichtungen einer schnellen Abführung derselben ins Blut ausgestattet sind.

Das lymphoide Gewebe ist schließlich, wie ich verschiedentlich gezeigt habe, als ein Grundgewebe aufzufassen, in dem die Sekundärknötchen entstehen und vergehen. Es steht den aktiven Sekundärknötchen als ein mehr ruhendes Gewebe gegenüber. Es ist deshalb berechtigt, es mit einem besonderen Namen zu belegen, wie ASCHOFF (1926) getan hat. Ob es außerdem noch eine besondere Funktion hat, kann heute noch nicht entschieden werden.

ZUSAMMENFASSUNG DES ZWEITEN ABSCHNITTS

Bei 173 Kaninchen wurde Staphylokokkenvaccine, Benzol-Olivenöl, Terpentinöl oder Arsen in nicht tödlicher Dosis subkutan in den rechten Unterschenkel injiziert. Bei 24 weiteren Kaninchen wurden die Arsenversuche WAETJEN's (1925) mit tödlichen Dosen nachgeprüft. Während sich bei den Arsentieren an der Injektionsstelle bei der Sektion außer geringen Nekrosen keine besonderen Veränderungen fanden, entwickelten sich bei den übrigen Tieren ausgedehnte Abszesse. Der rechte regionäre Popliteallymphknoten reagierte (mit Ausnahme der mit tödlichen Dosen injizierten Arsentiere) nach anfänglichen regressiven Veränderungen mit einer lymphatischen Hyperplasie, deren Stärke bei den verschiedenen Experimenten variierte. Auch das anderswo in den Organismus eingelagerte lymphatische Gewebe nahm, wenn auch nur in geringem Maße, an diesen Veränderungen teil, wie sich an dem linken Popliteallymphknoten und der Milz feststellen ließ.

Bei den Arsentieren fanden sich die stärksten regressiven Veränderungen. Bei den mit nicht tödlichen Dosen injizierten Tieren folgte hierauf sehr bald eine lymphatische Hyperplasie, die aber nur sehr geringfügig war und mehr eine Regeneration der zerstörten Gebiete darstellte. Da das Arsen sicher sehr schnell resorbiert wurde und sich an der Injektionsstelle keine Eiterung entwickelte, wirkte es auf die Lymphknoten als ein einmaliger, kurzdauernder, starker Reiz.

Bei den anderen Tieren entwickelten sich aber Abszesse, die zu den injizierten Reizmitteln als ein weiterer Reiz auf den regionären

Lymphknoten hinzukamen. Während bei den Staphylokokkentieren anscheinend nur geringfügige regressive Veränderungen auftraten und hier das Wachstum des lymphatischen Gewebes sogleich einsetzte, war bei den Benzol- und Terpentinöltieren eine kräftige Schädigung des lymphatischen Gewebes sichtbar, die sich einerseits durch das reichliche Auftreten von retikulären Zellen in den Sekundärknötchen, im lymphoiden Gewebe und in den Sinus, und andererseits dadurch ausdrückte, daß hier die reticuloendotheliale Reaktion viel länger andauerte, und die lymphatische Hyperplasie viel später in Erscheinung trat, als bei den anderen Versuchen. Die lymphatische Hyperplasie entwickelte sich um so später, je später die Eiterung ihren Höhepunkt erreichte (Tabelle 1). Die Injektionen von Staphylokokkenvaccine, Olivenöl-Benzol und Terpentinöl wirkten also als länger dauernde Reize. Ob das Benzol und Terpentinöl selbst außer schädigenden Einflüssen auch noch andere Wirkungen auf das lymphatische Gewebe hatten, ist zweifelhaft. Wahrscheinlich verdankte die lymphatische Hyperplasie hier ihren Ursprung allein dem resorbierten Eiter.

a) Die Lymphopoëse (Taf. XI). Die Entstehung der Lymphocyten stimmte bei allen 4 Experimenten weitgehend überein. Eine gewisse Zeit nach der Injektion traten zunächst große Lymphocyten auf, die sich schnell vermehrten. Sie bildeten sich ganz diffus überall im lymphatischen und lymphoiden Gewebe und, wenigstens bei den Staphylokokkentieren, auch in den Sinus. Die meisten großen Lymphocyten bildeten sich jedoch um die Blutgefäße herum. Die ersten großen Lymphocyten entstanden bei allen Experimenten direkt aus undifferenzierten Mesenchymzellen, indem diese stark basophil wurden, anschwollen und sich mit Abrundung ihrer Ausläufer ablösten. Die großen und die aus ihnen hervorgehenden mittelgroßen und kleinen Lymphocyten teilten sich dann durch Mitose, wobei die Teilungsfähigkeiten mit der Größe der Zellen abnahm. Eine Entstehung von Lymphocyten aus ausdifferenzierten Reticuloendothelien oder eine Entwicklung von kleinen zu großen Lymphocyten konnte nirgends beobachtet werden.

Wir können somit bei der Lymphopoëse eine homoioplastische und eine heteroplastische unterscheiden. Unter physiologischen Bedingungen, wo der Lymphocytenverbrauch nur gering ist, mag die

Vermehrung durch Mitose den Lymphocytenbedarf decken. Bei erhöhten Anforderungen entstehen sie aber aus den undifferenzierten Mesenchymzellen.

Die neugebildeten Lymphocyten wanderten teils durch die Sinus und teils durch die Venen des lymphoiden Gewebes ins Blut aus. Die stärkste Lymphocytenemigration wurde dann beobachtet, wenn die Zahl der großen Lymphocyten bereits wieder im Abnehmen begriffen war und die lymphatische Hyperplasie gerade ihren Höhepunkt erreicht hatte oder kurz hinterher (Tabelle 1). Sie fand um so später statt, je später sich die lymphatische Hyperplasie entwickelte. Mit ihr ging auch eine Lymphocytenvermehrung im Blut parallel, die ihren Gipfel kurz nach der stärksten Lymphocytenemigration erreichte.

b) Die Histogenese der Sekundärknötchen und ihr Schicksal (Abb. 47). Die Entwicklung der lymphatischen Hyperplasie stimmte ebenfalls bei den 4 Experimenten weitgehend überein. Sie ging mit einer diffusen Wucherung los, wobei sich die vorhandenen Übergangssekundärknötchen im lymphoiden Gewebe auflösten. Am Rande der Rinde wurden nun sehr bald solide Sekundärknötchen neugebildet, und zwar die meisten bei dem Arsenversuch, bei welchem auch der stärkste Zerfall stattgefunden hatte. Während die Wucherung bei den Staphylokokkentieren, bei welchen sich die stärkste lymphatische Hyperplasie entwickelte, zu einer diffusen lymphoiden Hyperplasie führte, bildeten sich bei den übrigen Versuchen von Anfang an mehr knötchenförmige Anhäufungen von lymphoidem Gewebe, d. h. Pseudosekundärknötchen, die ihren Ursprung teils aus den regressiv umgewandelten Sekundärknötchen und teils aus den neugebildeten soliden Sekundärknötchen nahmen. Erst als die lymphatische Hyperplasie ihrem Höhepunkt nahe war, bildeten sich FLEMMING'sche Sekundärknötchen oder sog. Keimzentren, mit deren Entwicklung die Hyperplasie ihren Höhepunkt erreichte. Sie blieben dann bis ans Ende des Versuches in voller Ausdehnung erhalten, indem sie ständig vergingen und neugebildet wurden.

Die soliden und FLEMMING'schen Sekundärknötchen entstanden dadurch, daß sich an bestimmten Stellen im lymphoiden Gewebe um Arteriolen und Kapillaren herum große Lymphocyten aus den undifferenzierten Mesenchymzellen bildeten, die sich bei den ersteren sehr schnell in kleine Lymphocyten umwandelten, während sie sich bei den letzteren nur zu mittelgroßen Lymphocyten weiter entwickel-

ten. Während die soliden Sekundärknötchen stets primäre Bildungen im lymphoiden Gewebe waren, entstanden die FLEMMING'schen entweder primär im lymphoiden Gewebe oder sekundär in soliden Sekundärknötchen, indem die schnelle Umwandlung der großen Lymphocyten in kleine aufhörte und sie bei der Bildung von mittelgroßen stehenblieben. Die soliden Sekundärknötchen konnten aber auch weiterwachsen und zu Pseudosekundärknötchen werden, oder sich diffus im lymphoiden Gewebe auflösen.

Die FLEMMING'schen Sekundärknötchen oder die sog. Keimzentren wandelten sich meist regressiv in Übergangssekundärknötchen um, womit ihre Entwicklung für gewöhnlich abgeschlossen war. In seltenen Fällen konnten sie aber kleine Lymphocyten bilden und sich direkt in Pseudosekundärknötchen verwandeln und zwar dann, wenn die ersten FLEMMING'schen Sekundärknötchen auftraten und die kleine Lymphocyten bildenden Reize zwar im Erlöschen, aber noch vorhanden waren. Die Übergangssekundärknötchen gingen meist im lymphoiden Gewebe auf. Traf sie aber ein neuer Lymphocyten bildender Reiz, konnten sie auch wieder zu wachsen anfangen und zu Pseudosekundärknötchen werden. Die Pseudosekundärknötchen lösten sich schließlich dadurch im lymphoiden Gewebe auf, daß ihre Lymphocyten abwanderten, und sich neue Sinus in ihnen bildeten.

c) Die Bedeutung der Sekundärknötchen und des lymphoiden Gewebes. Wie aus unseren Untersuchungen hervorgeht, sind die soliden Sekundärknötchen Orte besonders lebhafter Lymphocytenvermehrung, in denen die Umwandlung der großen Lymphocyten in kleine sehr schnell vor sich geht. Sie finden sich immer bei großem Bedarf an kleinen Lymphocyten, z. B. in der frühesten Jugend, wo ein starkes Wachstum der lymphatischen Organe stattfindet, und zu Anfang einer lymphatischen Hyperplasie. Sie wachsen je nach der Stärke des lymphocytenbildenden Reizes entweder zu Pseudosekundärknötchen oder zu diffusem lymphoiden Gewebe aus.

Die Pseudosekundärknötchen sind nichts weiter als knötchenförmig angeordnetes lymphoides Gewebe, d. h. Ansammlungen von blutreifen Lymphocyten, die teils durch die Sinus und teils durch die Venen auswandern. Die für das lymphoide Gewebe charakteristischen Venen sind in ihnen besonders gut entwickelt. Sie sind somit mit allen Vorrichtungen einer schnellen Abführung der Lymphocyten ins Blut ausgestattet.

Das diffuse lymphoide Gewebe ist für gewöhnlich als Grundgewebe aufzufassen, in dem die Sekundärknötchen entstehen und vergehen. Es steht also den aktiven Sekundärknötchen als ein mehr ruhendes Gewebe gegenüber. Bei starken lymphocytenbildenden Reizen kann es aber den ganzen Lymphknoten ausfüllen und ist dann Ausdruck einer hochgradigen Wucherung von Lymphocyten überall in den Lymphknoten.

Die FLEMMING'schen Sekundärknötchen oder die sog. Keimzentren sind, wie die soliden Sekundärknötchen, Orte lebhafter Lymphocytenvermehrung. Doch führt diese nicht zur Bildung von kleinen Lymphocyten, wie FLEMMING angenommen hatte (1885), sondern nur zur Entwicklung von mittelgroßen Lymphocyten, die an Ort und Stelle liegen bleiben. Ihr Schicksal ist für gewöhnlich, sich in Übergangssekundärknötchen umzuwandeln, welche nichts weiter als zur Ruhe gekommene FLEMMING'sche Sekundärknötchen sind und ihre Auflösungsphase darstellen. Die FLEMMING'schen Sekundärknötchen fanden sich gerade dann, wenn die Bildung kleiner Lymphocyten aufgehört hatte und die Lymphocytenemigration ins Blut im Abnehmen begriffen war und hinterher (Tabelle 1). Während FLEMMING's Theorie somit abgelehnt werden muß, spricht nichts gegen meine 1929 (a) aufgestellte Reservedepottheorie, nach welcher die hellen Zentren der FLEMMING'schen Sekundärknötchen Reservedepots von mittelgroßen Lymphocyten sind, die hier im Sinne einer Regeneration im Überschuß gebildet wurden. Einige Bedenken, welche ich früher gegen diese Theorie hatte, konnten durch diese Arbeit beseitigt werden. Ob den FLEMMING'schen Sekundärknötchen außerdem noch eine besondere Funktion innewohnt, wie von verschiedenen Forschern angenommen wird, kann heute noch nicht entschieden werden. Bevor wir weiteres darüber aussagen können, müssen wir wohl erst mehr über die Funktion der Lymphocyten wissen.

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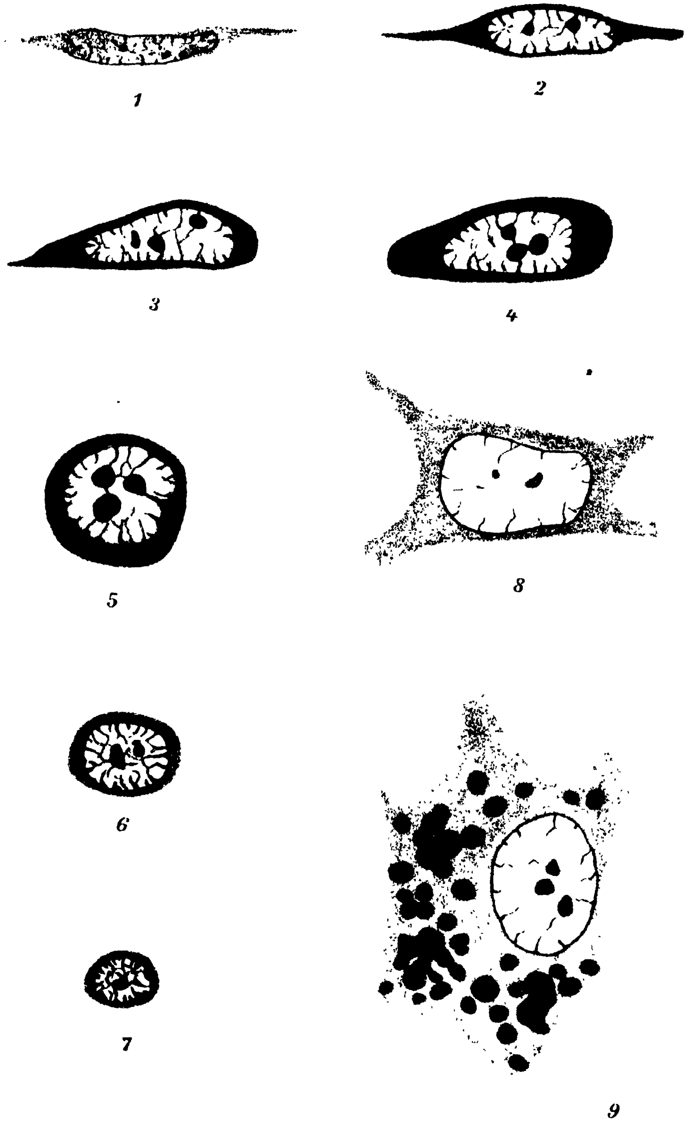
TAFELERKLÄRUNG

Tafel XI

Die Entstehung der Lymphocyten. Methylenblau-Eosin. 1 = undifferenzierte Mesenchymzelle, 2–4 = prolymphocytäre Stadien, 5 = großer, 6 = mittelgroßer und 7 = kleiner Lymphocyt, 8 = ruhende und 9 = aktive Reticuloendothelzelle. 1800 ×.



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THE PATHOLOGY OF EXPERIMENTAL DERMAL PNEUMOCOCCUS INFECTION IN THE RABBIT

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PLATES 2 AND 3

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The development and progression of the morphological changes in the human pneumonic lung are still incompletely understood, a fact partly explainable by the circumstance that pneumonic lesions, lobar in distribution, can be produced in animals only with difficulty. In previous papers (1, 2) one of us has described an experimental disease brought about by infecting rabbits intradermally with pneumococci (Type I). Although in this infection the focus is in the skin, there exist certain clinical and immunological similarities with lobar pneumonia in man; moreover, the visible location of the principal lesion is a definite aid to experimental studies. Observations made in the course of other experiments of this nature suggested that a study of the morphological alterations, both gross and microscopical, might lead to a better understanding of the fundamental phenomena of pneumococcic infection irrespective of the site of the lesion.

It may be recalled from an earlier report (1) that if a normal rabbit is given an intradermal inoculation of virulent pneumococci on the flank a local infection develops in the course of several hours. The lesion soon begins to spread ventrally and within 24 hours occupies a large area extending from the site of inoculation across the ventral midline. The infection is characterized particularly by the production of massive edema.

The course of the development of the lesion, as previously reported (1), may be summarized as follows:

After the intradermal injection of 0.2 cc. of an 18 hour blood broth culture there is at first a latent period of some 2 hours. Near the site of inoculation there then appear signs of early inflammation; these are characterized at first by conges-

tion of vessels and capillaries; the sharp vascular outlines are then lost and an area 1 to 2 cm. in diameter becomes slightly edematous and shows a trace of orange-red color. As more edema develops the accumulation of fluid is most pronounced at the ventral border. The lesion spreads ventrally at a rate of 2 to 3 cm. an hour, the heightened color being apparently preceded by the movement of fluid. At 10 to 12 hours the edematous band has usually extended to the ventral midline. More and more serous material accumulates in this region until the entire area is tense and swollen. Within 18 to 24 hours a certain degree of induration is present and this increases gradually until at 48 hours the involved tissue is firm and relatively non-elastic.

At 24 hours the lesion usually occupies a strip 2 to 3 cm. wide on the flank increasing to a width of 4 to 6 cm. at the ventral midline. Beginning at 24 to 30 hours there is a marked contraction of the involved tissue, and large irregular folds develop over the more ventral section. The color of the lesion varies from a pale to a moderately bright orange-red. In the infections which appear to be the more severe and fatal the involved skin often takes on a hemorrhagic character frequently evidenced by scattered ecchymoses or by widespread hemorrhage. Some frank necrosis with secondary infection may take place, the incidence varying with the amount of trauma to which the tissue has been subjected.

Methods

In order to study the histological development of this dermal lesion, a series of normal animals were injected intradermally with 0.2 cc. of an 18 hour blood broth culture of Pneumococcus Type I. The site of injection in each instance was at a point on the animal's flank approximately 8 cm. dorsal to the ventral midline. The development of the lesions and the concomitant temperature reactions were then carefully followed.

Except in one instance the animals were killed at various intervals after infection by inducing air embolism. The entire skin lesion, including the underlying muscle and peritoneum, was removed, lashed to cardboard to prevent distortion and by a longitudinal incision divided into two equal pieces. One was fixed in Zenker-formol and the other in Zenker's fluid to which 5 per cent of glacial acetic acid had been added. Multiple blocks, suitable for study of the entire extent of the lesion were embedded in paraffin, sectioned, and stained with hematoxylin-eosin, eosin-methylene blue, and Mallory's phosphotungstic acid hematoxylin. Stains for bacteria were made according to the method of Brown (3).

HISTOLOGICAL OBSERVATIONS

The earliest lesion to be examined was removed 2 hours after infective inoculation and before any elevation of the animal's temperature had taken place.

The only external evidence of disease was a soft, pale area of very slight swelling about the point of inoculation. Histological examination shows a marked accumu-

lation of edema fluid in the corium and in the loose adipose and connective tissue between corium and muscle. A moderate number of polymorphonuclear leukocytes are present although fibrin is not seen nor are erythrocytes in evidence in any considerable number. The muscle layer and corium show scattered infiltration with leukocytes. Blood vessels, particularly the smaller ones, are congested, dilated, and appear more numerous than in the normal skin. The lymphatics also appear larger and more numerous but contain few leukocytes and no fibrin. Study of the borders of the lesion indicates clearly that extension of the edema fluid takes place in advance of infiltration with inflammatory cells. No evidence of tissue necrosis can be made out. Nearer the center of the lesion, presumably in relation to the site of inoculation, a marked accumulation of mononuclear wandering cells has taken place in and around the adventitia of the blood vessels. A photograph of the lesion at this stage is reproduced in Fig. 1. This shows the early histological development with edema distending the corium and subcorial space. Fig. 2 shows the uninvolved tissue just peripheral to the lesion.

The progression of the histological alterations was next studied in a lesion taken at 5 hours.

This animal's temperature had risen to 104°F., an elevation of a little over a degree from the normal. The involvement at this time measured 2.5 by 3.5 cm. the latter being the dorsoventrad dimension. This area was definitely edematous, a feature most prominent at the ventral border. The color except for the most ventral portion was a faint orange-red and the consistency very soft.

At this time the histological picture generally parallels that described at 2 hours, but all features are more marked. The loose connective tissue fibers of the corium and panniculus adiposus have been widely separated from each other by the edema fluid, and this has resulted in a structure which, on superficial examination, appears not unlike the areolar structure of pulmonary parenchyma. The relatively thin tissue layer has thus been converted into the outstanding anatomical structure. The cellular infiltration has extended into the deeper layers of the corium but as yet is not a marked feature. The dilatation of the lymphatics and the increase in size and number of functioning vessels present a picture similar to that seen in the earlier specimens. Despite the size and rapid progression of the lesion, the response in the form of cellular exudate is only moderate in degree. Necrosis of tissue cannot be detected, nor can any deposition of fibrin be observed. Although dilated lymphatics and engorged blood vessels form a prominent feature at this stage, even more so than in the earlier lesion, no evidence of thrombosis can be found.

The next tissue studied was removed from an animal 8 hours after infective inoculation.

The rabbit's temperature had risen to 105°F. The lesion occupied an area 2.5 by 8.5 cm. extending to and just beyond the ventral midline. The entire zone was soft and edematous, a change most pronounced at the more ventral projection. Near the point of original inoculation the color was a very bright orange-red but this shaded off gradually until the ventral third of the area was of the same color as the surrounding tissue. At this time sections show that there had been a distinct shift in the predominant cell type of the inflammatory reaction. The perivascular grouping of mononuclear phagocytic cells is a striking feature, many cells of this type having also taken part in the general cellular response. Edema is still the main feature of the lesion and has completely separated corium from muscle. Infiltration, both of polymorphonuclear and mononuclear cells, is steadily increasing in degree and extends, at this time, well into both corium and muscle layers. Still no hemorrhage or necrosis is present. The degree of lymphatic dilatation does not appear to be altered but more small blood vessels, greatly engorged, are appearing in the corium.

The further progress of the pathological processes was studied in a lesion removed at 28 hours.

The animal's temperature had been over 104°F. for approximately 20 hours, with a maximum high level of 105.5°F. The lesion at this time occupied a strip 3 cm. wide extending downward from the point of inoculation to the ventral midline where the involvement measured 8 cm. in diameter. The entire area was greatly swollen, an alteration accentuated by the formation of huge folds caused by the contraction of the skin. The lesion was rather firm, especially in the area near the site of inoculation. The color varied from a pale to a bright orange-red. No macroscopic necrosis or hemorrhage was apparent. Microscopically, the sections show at the site of maximum involvement, near the juncture of upper and middle third of the lesion, the earliest evidence of death of the cells forming the exudate. Here groups of acidophilic mononuclear and polymorphonuclear leukocytes are seen. Peripheral to this reaction, the picture previously described of corial and subcorial edema associated with mild inflammatory infiltration is encountered. Perivascular cell infiltration is still marked and the large number of newly formed or newly functioning capillaries is most striking. The lower edge of the lesion, on the other hand, as the most recently involved, presents changes not unlike those seen in the preparations previously described. Here, the edema, both in the subcorial space and in the inferior layers of the corium, is by far the most prominent change. Inflammatory exudate is sparse and diffuse.

As an example of the fully developed lesion, tissue was studied 2½ days after infection.

This animal died under observation after a typical course of the disease associated with high temperature. The tissue was excised and fixed immediately

after death. The lesion occupied an area of much the same size as that last described. The edema was massive, firm, and circumscribed. A bright orange-red color, most pronounced in the early phases, was succeeded at 45 hours by ecchymoses which rapidly progressed to hemorrhagic necrosis. At this time a radical alteration appears in the histological picture at the oldest part of the lesion, situated well toward the dorsal end. Here, in addition to the edema previously described, fairly dense infiltration composed of mononuclear and polymorphonuclear leukocytes is found in the lower layers of corium and the strata of the upper muscle layers. Groups of necrotic inflammatory cells are occasionally encountered although necrosis of parenchyma is not apparent. Diffuse hemorrhagic infiltration is striking in certain areas. Peripheral to this well established lesion, there are regions presenting rather less activity, where the edema is most prominent and the cellular infiltration not marked. A large number of new or newly functioning capillaries and even larger vessels have appeared, many surrounded by a collar of mononuclear cells. Some proliferation of fibroblasts has taken place in the lower corium. The epithelial surface presents a number of small areas where the cells have undergone a degenerative change overlying a base of coagulated exudate containing roughly parallel rows of polymorphonuclear and mononuclear leukocytes. Subepithelial lymphatics are greatly distended. Inflammatory infiltration extends well up to the epithelium and down to the second layer of muscle. A certain number of muscle fibers appear necrotic. Thrombosis of both large and small veins is well marked in this preparation, a condition presumably accountable for the marked hemorrhage present.

The last example studied was that of an advanced lesion, removed at 99 hours after infection.

This presented essentially the same gross pathological picture as that just described, except that the hemorrhagic condition was never so prominent. Microscopically, this lesion shows a picture unlike that seen at any other period studied. The process is one of repair and of reaction to foreign material represented by groups of cellular débris. Much edema is still present and there is a massive infiltration with leukocytes, predominantly mononuclear in type. Many new vessels and young active fibroblasts are seen, a change particularly marked around the pockets of necrotic material. Thrombosis is a marked feature. The extensive edema and scattered cellular infiltration, so marked in the earlier slides, is not apparent in this later preparation. Figs. 3 and 4 show low and high magnifications of a section of a mature lesion.

These examples show the course of development and maturation of the lesion. Studies on the histological changes occurring during resolution are now in progress and will be presented in connection with certain data bearing on the recovery process.

DISCUSSION

In order to relate these observations to the problem of the early morphological alterations in lobar pneumonia, it is first necessary to consider in some detail the changes which take place in the human lung. Briefly, these changes have been described as engorgement, red hepatization, gray hepatization, and resolution.

The first stage, that of engorgement, is not commonly seen at autopsy and the descriptions for the most part have been derived from study of tissue contiguous to a pneumonic process in the direction in which the pneumonic process is presumed to be spreading. Here engorged vessels may often be found. More commonly, however, the only evidence of progression is the accumulation, in the alveoli, of edema fluid containing a few polymorphonuclear leukocytes. Blake and Cecil (4), as well as Winternitz and his coworkers (5) have called attention to the dilated lymphatics in experimental pneumococcus pneumonias in animals. Permar (6), on the other hand, studied the lesion of experimental pneumonias sooner after infection than was done by the workers just mentioned and pointed out that the most striking change was the presence of intraalveolar fluid in the absence of lymphatic involvement. Loeschcke (7), in a detailed study of the histological alterations in the human pneumonic lung, has called attention to the prominence of edema formation in the early and advancing lesions.

Comparing these changes, considered to be the earliest manifest in the pneumonic lung, with the alterations observed in the infected skin of the rabbit a remarkable similarity is immediately evident. The accumulation of a relatively cell-free exudate and the presence of dilated lymphatics, dilated and engorged capillaries, and an intact supporting structure are all found in the dermal infection. The progression of the infection by the production of edema fluid, presumably containing the infecting bacteria, would suggest strongly that a similar mechanism may obtain in the pneumonic lung.

The stage of red hepatization is only rarely encountered in lobar pneumonia. It is usually described, however, as being characterized by a deep red color, presumably due to dilated and engorged capillaries of the alveolar wall. The exudate stains well and contains a considerable number of polymorphonuclear leukocytes and a certain amount of fibrin, although the latter is a variable factor. The interstitial tissue is still intact and the lymphatics are dilated, containing a certain number of inflammatory cells.

Turning to the lesion observed in the rabbit skin, the same general pathological alterations are found. Vascular congestion is a marked

feature and the inflamed area appears red on gross inspection. The exudate near the site of inoculation now contains a larger number of inflammatory cells, mostly polymorphonuclear leukocytes, as well as a few mononuclears. The number of erythrocytes is extremely variable. Fibrin may be present but it is not a striking feature. The lymphatics are markedly dilated and may contain a moderate number of leukocytes. As in the lung no evidence of damage to the supporting structure is encountered. At the periphery of the progressing lesion, the changes are identical with those of the initial reaction present throughout when the tissue is examined a few hours after inoculation. Exactly the same condition may be encountered in the human pneumonic lung, where, at the periphery of the consolidated tissue, areas may be found in which the alveoli contain edema fluid with a few cells and the vessels are greatly engorged, a picture presumed to be widespread early in the disease. As in the lung no necrosis is to be found, so also, the interstitial tissue of the rabbit skin is quite intact.

The third stage of lobar pneumonia, that of gray hepatization, is the one most frequently encountered at autopsy. Here, the fibrin of the exudate is commonly most dense and shows a tendency to shrink away from the alveolar walls. The cellular exudate, composed for the most part of polymorphonuclear and mononuclear leukocytes, stains poorly and is presumed to be largely necrotic. The small vessels in the walls of the alveoli are inconspicuous and may contain no blood whatever though this can hardly be stated to be the rule. A number of mononuclear phagocytic cells have appeared by this time, and many contain droplets of fat or fine granules of hemosiderin.

The comparison with the more advanced lesion of the rabbit dermal infection is a striking one. Here, corresponding to the pneumonic lung, one finds that the oldest exudate is necrotic and that mononuclear phagocytes are engaged in carrying away various broken-down products. Capillary engorgement is by no means so marked and although many new vessels have appeared they are in association with granulation tissue, a change easily understood when one considers that in the skin the exudate has no ready route of exit. The removal of the exudate must take place more slowly and a certain amount of organization is bound to occur. Necrosis of dermal tissue is, however, not a feature and in this respect the analogy with the pneumonic lung still holds. This analogy is also true in the case of the fibrin which is

much more dense in both of the lesions at this stage. One feature deserves especial attention and that is the occurrence of hemorrhage. In the dermal lesion this is fairly striking in certain of the more advanced stages but it has not been commonly described in discussion of the pathology of lobar pneumonia. Loeschcke (7) has recently called attention to the occurrence of intraalveolar hemorrhage in the later stages of lobar pneumonia. This author explains his finding by the shrinkage of the fibrin plugs away from the alveolar walls, allowing a decrease of pressure on the alveolar side of the membrane and consequent rupture of the weakened vascular wall with resultant outpouring of red blood cells into the alveoli. It is undoubtedly true that hemorrhagic areas are frequently observed in the pneumonic lung at autopsy and a detailed microscopic study of a limited amount of material available to us has revealed a surprising amount of intraalveolar hemorrhage in areas of the lung known from clinical data to represent the older pathological processes. In such preparations thrombosis of the finer vessels appears to give rise to this phenomenon and here again the same condition is found in the skin lesion.

In recapitulation, it may be said that the predominant change in the rabbit skin, infected with pneumococci, appears to be the production of an extraordinary volume of edema fluid. This fluid, carrying pneumococci, seems to spread rapidly, filling the interstices of the connective tissue framework. The direction which the fluid takes is influenced principally by gravity. Following the production of fluid there appears a cellular exudate, predominantly polymorphonuclear but in the later stages containing also many mononuclear cells. As the age of the lesion increases more fibrin is seen and eventually the oldest exudate becomes necrotic, although at the border the relatively pure edematous lesion is still present. Hemorrhage may be a late manifestation. Since changes of the same general nature may be observed in the pneumonic lung, it is of some interest to consider the likelihood that the pulmonary disease may extend by a similar mechanism, that is to say, by the production of an infectious edema fluid near the hilus with secondary spread of this fluid by force of gravity and by the churning action of the moving alveolar walls.

The chief immediate significance of these findings is that they establish the fundamental similarity of the pathological processes of the

rabbit dermal disease and those of human lobar pneumonia. This similarity has importance for work to be reported later on factors which affect the development, progression, and localization of the rabbit lesion, as well as in relation to the method that has been suggested for the comparison of the curative properties of antipneumococcic sera by use of this experimental infection (8).

SUMMARY

1. The pathology of the experimental dermal pneumococcic infection in the rabbit is described in detail and the findings are compared with the histological alterations seen in the human pneumonic lung. There would appear to be a basic similarity of the lesions in both tissues.

2. A copious production of edema fluid is the outstanding characteristic of the early lesion. It occurs prior to any significant cellular change. In the spreading lesion an infiltration of the tissues with fluid precedes any other sign of reaction between tissue and microorganism. It seems likely that the advancing fluid carries with it the infecting organisms and inoculates all tissues which it reaches. The resulting infection seems not to take place by an active invasion of microorganisms but by a progressive inoculation from an infected fluid.

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EXPLANATION OF PLATES

Plate 2

Fig. 1. Photomicrograph of a section of a lesion from the skin of a rabbit 2 hours after infection with Pneumococcus, showing marked edema of corium and subcorial space associated with slight cellular infiltration. × 65. Phosphotungstic acid hematoxylin.

Fig. 2. Same section, showing relatively unaffected tissue beyond the periphery of the lesion depicted in Fig. 1. × 65. Phosphotungstic acid hematoxylin.

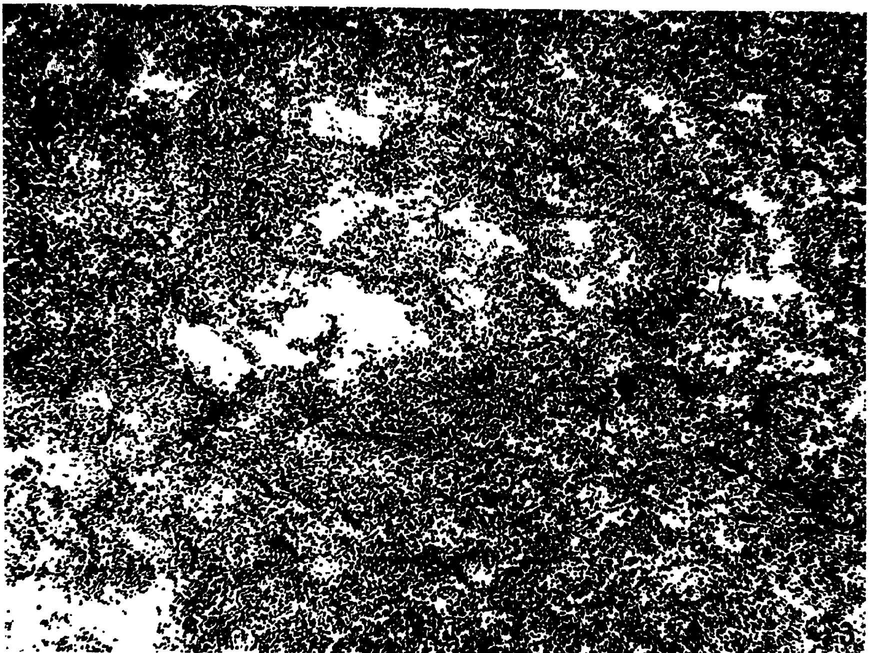
PLATE 3

Fig. 3. Low power photomicrograph of corium showing the intense inflammatory infiltration in the mature lesion. × 65. Eosin-methylene blue.

Fig. 4. Same section, with higher magnification, indicating the marked edema, the character of the cellular infiltration, and the separation of connective tissue fibrils. × 550. Eosin-methylene blue.

Photographed by Louis Schmidt

(Rhoads and Goodner: Dermal pneumococcus infection)



Photographed by Louis Schmidt

(Rhoads and Goodner: Dermal pneumococcus infection)

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DECOMPOSITION OF THE CAPSULAR POLYSACCHARIDE OF PNEUMOCOCCUS TYPE III BY A BACTERIAL ENZYME

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It is now generally recognized that one of the important differences between the two variants of Pneumococcus, the so called R and S forms, is the presence around the latter of a capsule which has been shown to contain a complex polysaccharide; the type specificity of Pneumococcus and the virulence of the S cells are associated with the presence of this capsular polysaccharide which is referred to as the soluble specific substance; the chemical structure of the capsular polysaccharides has been shown to vary from one type of Pneumococcus to another (1); in fact, they are as chemically distinct one from the other as they are serologically specific.

The fact that the capsular polysaccharides of Pneumococcus—and not some impurities carried along with them—are themselves the substances responsible for type specificity, has been shown by the disappearance of their reactivity in the specific antisera after they had been subjected to acid hydrolysis. However, this treatment is a fairly drastic one and may have affected at the same time the hypothetical impurities. It was thought that such an objection would be removed if the polysaccharide could be split by the milder action of an enzyme.

It was interesting also to determine whether the addition of such an enzyme to a medium seeded with the encapsulated pneumococci would effect a dissolution of the capsule. From this point of view the observations of Toenniessen (2) are of special interest. He found that when *Bacillus vulgatus* was seeded together with encapsulated Friedländer bacilli, the latter organisms grew deprived of their capsule. Finally, we had in view experiments to determine whether the injection into the animal body of an enzyme capable of decomposing the cap-

sular polysaccharide would in any way alter the course of experimental infection induced by Pneumococcus of the same type as that from which the specific substance had been derived.

A systematic search for specific enzymes of this order has been carried on in this laboratory for several years. A number of enzymes from animal and plant sources, known to be active in the hydrolysis of simpler carbohydrates, were tested, but none of them were found capable of attacking the polysaccharides of pneumococcus origin. In addition, cultures of various molds, yeasts, soil actinomycetes, and bacteria, many of which were known to decompose cellulose and other complex carbohydrates, were tested without success.

Recently, however, a microorganism has been isolated, and an enzyme extracted from it, both of which are capable of decomposing the capsular polysaccharide of Type III Pneumococcus (3). The object of this first paper is to describe the technique of isolation, the morphological, cultural, and biological characteristics of the organism; and to define the nature and mode of action of the enzyme derived from the bacterial cells.

EXPERIMENTAL

1. Inoculation Material.—As mentioned previously, many organisms known to decompose actively various polysaccharides were tried and were found unable to decompose the capsular polysaccharides of Pneumococcus. Since these specific substances have many of the properties of hemicelluloses, an attempt was made to search in a natural environment for organisms possessing the capacity to split complex substances of this nature. It was thought that those locations where large amounts of organic materials,—especially belonging to the group of "hemicelluloses,"—accumulate and undergo decomposition, were the most likely to harbor the desired organisms. Among the materials tried were leaf mold, composts (of corn cob, rye straw, sphagnum, oak leaves, etc.), farm manure, soils rich in organic matter (different peat soils and soils heavily manured).[1] From a

[1] Many of these materials and especially the peat samples from the cranberry bogs of New Jersey were supplied by Dr. S. A. Waksman and Dr. R. L. Starkey of the Department of Microbiology of the New Jersey Experiment Station to whom we extend our heartiest thanks for their courtesy and cooperation.

sample of soil from the cranberry bogs of New Jersey a microorganism has been isolated which is capable of decomposing the capsular polysaccharide of Pneumococcus Type III.

2. *Medium.*—The mineral medium used was based on one previously described for the isolation of cellulose-decomposing bacteria (4).

Ammonium sulfate (1 gm. per liter) was used as a source of nitrogen, dibasic potassium phosphate (2 gm. per liter) as buffering agent and source of phosphate, tap water supplied traces of the other mineral elements. The reaction of the medium was adjusted to the proper pH with HCl and NaOH. To this mineral solution, the capsular polysaccharide of Type III Pneumococcus was added in final concentrations varying from 0.001 to 0.2 per cent. This substance was the only source of organic carbon in the medium.

The soluble specific substance used in these experiments was prepared from a strain of Type III Pneumococcus by the method previously described (1, 5). This nitrogen-free preparation yields on hydrolysis a mixture of aldobionic acid and glucose. However, the molecule seems to be built up exclusively of aldobionic acid groups, the glucose appearing only as a secondary product of hydrolysis (6).

The use of this simple and specific medium was dictated by the following considerations.

(*a*) It was possible that the materials used for inoculation contained organisms *potentially* capable of decomposing the specific substance but for which other nutrients would act as more readily available sources of energy. Such organisms would attack the specific substance when deprived of any other food but leave it untouched in a complex medium. In fact, the production of an enzyme is often the result of what has been termed a "starvation" phenomenon. For example, Brown and Morris (7) found that the secretion of diastase by germinating barley seeds is inhibited by the presence in the medium of sugars which can be utilized directly by the growing plantlet. In a review on bacterial enzymes, Waksman has cited many similar examples which occur in the microbial world (8). Wortman (9) for instance, found that a certain bacterium had the power of excreting a starch-dissolving enzyme when starch was the only available food, but that no secretion of enzyme occurred if sugar or tartaric acid was offered to the organism along with the starch.

(*b*) The material used for inoculation was of course a mixture of a

great variety of microbial species. A medium containing the specific substance as sole source of carbon rendered conditions favorable only for these organisms capable of utilizing the specific substance itself or the products of its decomposition. This procedure afforded a means of rapidly eliminating a large number of irrelevant species.

(*c*) The ultimate object of the work was the preparation of an enzyme specifically directed against the capsular polysaccharide. For study of the action of the enzyme on the growth of Pneumococcus, and on the course of pneumococcus infection in experimental animals, it was especially desirable to have a preparation as poor as possible in proteolytic activity. From this point of view, it was expedient to use a mineral source of nitrogen instead of peptone or protein.

3. Technique of Isolation.—In order to eliminate as many as possible of the organisms which although unable to attack the specific substance may grow on the products of its decomposition or on the bacterial bodies, transfers were made as soon as growth could be detected in the cultures, in the hope that the first organisms to develop would be the ones attacking the specific substance and that in young cultures they would outnumber the others which could then be eliminated by diluting the inoculum.

4. Serological Method for Following the Disappearance of the Specific Substance.—The presence or absence of the specific substance in a culture was tested by the precipitin reaction:

0.5 cc. of fluid to be tested was added to 0.2 cc. of Type III antiserum[2] and the mixture brought to a volume of 1 cc. by the addition of salt solution. Since the precipitation test gives a positive result with a concentration of specific substance as low as 1:5,000,000, the absence of a positive precipitin reaction was interpreted as evidence of complete decomposition of the specific substance.

The Organism

1. Isolation.—The presence in a sample of soil from the cranberry bogs of New Jersey, of an agent capable of decomposing the Type III specific substance, is demonstrated in the following experiment.

[2] The Type III antipneumococcus serum used in these experiments was furnished through the courtesy of Dr. A. B. Wadsworth, Director of the Division of Laboratories of New York State Department of Health.

Experiment 1. The Effect of Environmental Conditions (pH, Temperature, Aeration) on the Decomposition of the Capsular Polysaccharide by an Agent Present in Peat Soil.—Different lots of basic mineral medium containing 0.002 per cent of Type III specific substance were adjusted to pH 4.5, 5.0, 5.5, 6.2, 6.6, 7.0, 7.8, and 8.5 Each one of these media was divided into three small Erlenmeyer flasks (25 cc. per flask) and into three Noguchi tubes (10 cc. per tube). Each of the tubes and flasks was inoculated with about 0.5 gm. of peat soil (from the cranberry bogs of New Jersey). The Noguchi tubes were incubated under anaerobic conditions (Brown jar) and the Erlenmeyer flasks under aerobic conditions. In each instance one set was kept at room temperature (about 22°C.), a second set at 37.5°C., and a third set at 54°C.

The cultures, in mineral media at different hydrogen ion concentrations and incubated at different temperatures under aerobic and anaerobic conditions, were tested from time to time for the presence of the specific substance. The specific precipitin reaction became negative first in the aerobic flask at pH 7.8, after 24 days incubation at 37.5°C. Within the following 10 days, the test became negative also in all the aerobic cultures at pH 6.2, 6.6, 7.0, and 7.8 at room temperature and 37.5°C. The specific substance did not disappear in the other flasks nor in the anaerobic tubes, even after 2 months incubation.

These results pointed to the existence of an agent capable of decomposing or removing from solution the specific substance between pH 6.2 and 7.8, at room temperature and 37.5°C., under aerobic conditions. That it was a living agent was shown by the fact that it could be transferred in series.

Since we had in view the use of this agent in the animal body, it was advisable to incubate the cultures at 37.5°C. in a medium of approximately neutral reaction; these conditions approach those present in the animal body and had been found in Experiment 1 to be close to the optimum for the activity of the microorganism.

Experiment 2. Attempts to Increase the Activity of the Culture by Repeated Transfers in Specific Medium.—Test tubes containing 10 cc. of the synthetic medium adjusted to pH 7.5 were inoculated with material from the aerobic flask in which the specific substance had first disappeared in Experiment 1. The incubation was carried on at 37.5°C., aerobically.

In this first transfer, the specific substance disappeared in 10 days. The culture was carried in the same medium for several months, transfers being made as soon as the specific substance had been decomposed,

in the hope of increasing the activity of the culture. In fact, after repeated transfers for 6 months, decomposition of 0.002 per cent of specific substance could be obtained regularly in 24 hours provided a young culture was used.

Experiment 3. Attempts to Purify the Mixed Culture by the Dilution Method.—A tube of synthetic medium was inoculated with a loopful of an active culture. 8 hours later stained films showed a fairly abundant growth of many different kinds of bacteria. At this time transfers were made into a series of tubes containing fresh mineral medium, using the following inocula: 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 cc.

After 18 hours incubation at 37.5°C. stained preparations of these cultures showed that growth had taken place in the first four tubes of the series. Serial transfers were immediately made from the fourth tube (0.0001 cc. inoculum) into fresh medium, using seedings varying from 0.1 to 0.00001 cc. of culture. 11 hours later growth was recognized (by microscopic examination) in the tube which received 0.001 cc. inoculum and from this culture serial transfers were again made.

The technique of purification by the dilution method was continued for 10 days. Each transfer was kept long enough to make sure that the active culture was carried along, as demonstrated by decomposition of the specific substance in 1 to 5 days, the time depending upon the size of the inoculum. Microscopic examination of a young culture of the last of the serial transfers, revealed the presence of only three morphological types of bacilli, a very small Gram-negative rod, a short plump Gram-negative rod, and a large Gram-positive rod. The impure culture thus obtained was plated in the hope of separating these three organisms, but unfortunately one of them was a spreader which overgrew the two other species.

Experiment 4. The Use of Gentian Violet in the Isolation of the Specific Organism from Mixed Cultures.—An active culture was seeded into mineral medium to which had been added 0.001 per cent of gentian violet in the hope that the bacteriostatic action of the dye would inhibit the development of the Gram-positive organism (10). 4 days later, the specific substance could no longer be detected in the culture containing gentian violet and microscopic examination showed the presence of only the two Gram-negative organisms, and a few large spores, apparently belonging to the smaller bacillus.

In the hope that the spore-bearing bacillus was the active form in the mixture, the following experiment was carried out.

Experiment 5. Isolation in Pure Culture of the Specifically Active Spore-Forming Bacillus by Heating the Inoculum at 70°C.—0.2 cc. of the culture just described was inoculated into two tubes of mineral medium containing 0.002 per cent of specific substance; one tube was then heated at 70°C. and the other at boiling temperature for 15 minutes.

After 7 days incubation at 37.5°C. the specific substance had been decomposed in the tube heated at 70°C. but it remained unaffected in the tube which had been boiled. Transfers made from the culture heated at 70°C. grew and decomposed 0.002 per cent of specific substance in 2 days; stained films of this culture showed what appeared to be a pure culture of a small, Gram-negative, spore-bearing rod, exhibiting metachromatic granules.

Young cultures of this organism plated on nutrient agar gave a pure growth of colonies which will be described later, whereas older cultures in the spore stage (5 to 6 days old) failed to grow on agar. Transfers were made from one of these colonies, and the Gram-negative bacillus in pure culture was carried for fifteen generations on blood agar from colony to colony. A colony from the fifteenth transfer was then inoculated into the mineral medium containing 0.002 per cent of Type III capsular polysaccharide. The latter was decomposed in 3 days, the culture showing again a pure growth of Gram-negative rods.

These experiments established the fact that the spore-bearing Gram-negative rod, and not some other agent carried along with it, is responsible for the decomposition of the specific polysaccharide of Type III Pneumococcus. For the sake of simplification, this culture will be referred to as the "S III bacillus."

2. Description of the Culture.—A morphological description of the organism is rendered difficult by its pleomorphism. In the mineral medium containing small amounts of specific substance (0.002 per cent), the organism appears as a minute Gram-negative bacillus, at times smaller than the Pfeiffer bacillus. In this medium also, spore formation takes place within the first 24 hours, and the cells are completely autolyzed by the 3rd day.

The cultural characters of the bacillus are much the same when grown in mineral medium containing larger amounts of specific substance (0.2 per cent); under these conditions the cells, however, are much larger and appear more resistant to autolysis.

The organism is Gram-negative in the mineral medium but harder to decolorize when grown in plain broth or on agar. At all stages, the cultures exhibit metachromatic granules, and in older cultures these granules appear as chains of minute coccus forms within the empty cell

membrane. The organism grows diffusely in peptone solution. In this medium sedimentation of the growth occurs after several days. On plain nutrient agar, free of dextrose, growth occurs in the form of small whitish colonies, 2 mm. in diameter, circular, slightly raised, umbilicated, with entire edge and fairly smooth surface. When grown in plain broth or in peptone solution, the organism appears as a fairly large bacillus, actively motile by means of peritrichous flagellae, the young cells measuring 2 to 3μ by 0.5μ. Short chains and especially diplo-forms are often observed. Organisms growing in this medium do not autolyze readily; in fact it is difficult to cause disintegration of the cells even by repeated freezing and thawing. Older cultures show the presence of elongated, thread-like involution forms and spores appear in 4 to 5 days. They are polar, oval, and very much larger than the rods. The heat resistance of the spores was measured in the following experiment.

Experiment 6. Heat Resistance of Spores of the S III Bacillus.—Tubes containing 5 cc. of casein peptone broth were inoculated with 0.2 cc. of a 5 day old culture of the bacillus. The inoculated tubes were heated at different temperatures for different lengths of time as indicated in Table I; the presence or absence of growth after 10 days incubation at 37.5°C. is indicated in the same table.

The results of Experiment 6 indicate that the spores of the organism resist heating for 30 minutes at 75°C., but are killed by boiling for 5 minutes.

The fermentative ability of the specific organism was determined by growth in the basic mineral media to which various sugars, glucosides, and alcohols were added in concentration of 1 per cent. No gas was formed in any of these media and slight production of acid was observed only in the presence of dextrin, galactose, lactose, maltose, salicin, and trehalose.

Of particular interest is the action of glucose on the growth of this organism. It has been found that the addition of this sugar, to an otherwise favorable medium, exerts a decidedly inhibiting action on the development of growth.

In all media thus far tested, the organism is strictly aerobic, no growth occurring under anaerobic conditions.

In media containing, in addition to the capsular polysaccharide, other nutrients such as peptone, the decomposition of the specific

substance is much delayed. The delayed decomposition under these conditions is probably attributable to the so called sparing action of the peptone, which serves as a more readily available source of energy. As an illustration of this fact, it may be mentioned that, in peptone broth and in mineral medium containing a concentration of capsular polysaccharide equivalent to that naturally present in an autolysate of a bouillon culture of Type III Pneumococcus, the rate of decomposition of the specific substance is much slower in both the peptone broth and the autolysate than it is in the mineral solution, although growth develops more abundantly in the peptone-containing media.

TABLE I

Heat Resistance of the Spores of the S III Bacillus

Time of exposure	Growth in casein peptone broth after heating inoculum as indicated						
	70°C.	75°C.	80°C.	85°C.	90°C.	95°C.	100°C.
min.							
5	+	+	+	+	+	+	−
10	+	+	+	−	−	−	−
15	+	+	−	−	−	−	−
20	+	+	−	−	−	−	−
30	+	+	−	−	−	−	−

\+ Refers to presence of growth after 10 days incubation.
− Refers to absence of growth after 10 days incubation.

It was of special interest to test the action of the organism on the specific polysaccharides of other types of Pneumococcus and on other polysaccharides of bacterial and plant origin.

Experiment 7. Specificity of the Action of the S III Bacillus.—The specific polysaccharides of Pneumococcus Types I, II, and III, Friedländer bacilli, Types A, B, and C (11), and *Hemophilus influenzae* Type *a* (12), and gum arabic were added to the basic mineral medium at pH 7.5, inoculated with 0.1 cc. of a young culture and incubated aerobically at 37.5°C. Decomposition of the different polysaccharides was tested by the serological method, using in each instance the specific antiserum, except in the case of gum arabic which was tested against Pneumococcus Type III antiserum (13) (see Table II).

The results of Experiment 7 emphasize the extraordinary specificity of the action of the organism on the specific polysaccharide of Type III

Pneumococcus. It is worth noting in particular that the organism does not decompose gum arabic even though this substance reacts in Type III antiserum.

The saprophytic nature of the S III bacillus is shown by the fact that 1 cc. of a young active culture may be injected intraperitoneally into a mouse without affecting the animal.

The Enzyme

The preceding experiments have established the existence of a microorganism which during growth in a synthetic medium breaks down the capsular polysaccharide of Type III Pneumococcus. It seemed possible that from cultures of this bacillus a soluble enzyme might be extracted which would decompose the specific substance in the absence of the bacterial cells.

TABLE II

Specificity of the Action of the S III Bacillus

Origin of the polysaccharide	Concentration of polysaccharide	Specific precipitin reaction of cultures after incubation at 37°C. for	
		1 day	30 days
	per cent		
Pneumococcus Type I	0.002	+++	+++
Pneumococcus Type II	0.002	+++	+++
Pneumococcus Type III	0.002	–	–
Friedländer's bacillus Type A	0.002	+++	+++
Friedländer's bacillus Type B	0.002	+++	+++
Friedländer's bacillus Type C	0.002	+++	+++
Hemophilus influenzae Type *a*	0.02	+++	+++
Gum arabic	0.01	+++	+++

+++ = marked precipitate formed in the corresponding specific antiserum.

– = negative precipitin reaction, indicating complete decomposition of the polysaccharide.

Experiment 8. The Extraction of a Soluble Enzyme Capable of Decomposing the Specific Polysaccharide of Pneumococcus Type III.—The mineral medium (pH 7.5) containing 0.01 per cent of Pneumococcus Type III specific polysaccharide was seeded with a heavy inoculum of the S III bacillus and incubated at 37.5°C.

The specific substance was completely decomposed after 24 hours incubation;

at this time, when microscopic examination showed large numbers of well formed cells with only a few spores, 10 cc. of the culture were removed and passed through an N Berkefeld filter. The filtrate was designated Preparation 3-*a*. After 5 days further incubation, microscopic examination of the original culture showed that all the cells were lyzed; the autolyzed culture was then passed through an N Berkefeld filter and the filtrate designated Preparation 3-*b*. Test tubes containing 1 cc. of a 0.001 per cent solution of specific substance (pH 7.5) received varying amounts of Preparations 3-*a* and 3-*b*; the mixtures were made up to a volume of 1.5 cc. and incubated at 37.5°C. for 18 hours in the presence of toluene to prevent any bacterial action. The precipitin test for the presence of specific substance was made after 18 hours incubation. The results of these tests are recorded in Table III.

TABLE III

The Decomposition of Type III Capsular Polysaccharide by Sterile Culture Filtrates of the S III Bacillus before and after Autolysis

Amount of filtrate	Specific precipitin reaction of mixtures of subtrate and culture filtrate	
	Before autolysis 3-*a*	After autolysis 3-*b*
cc.		
0.5	+++	−
0.1	+++	+
0 (control)	+++	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus sign indicates no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

The results of Experiment 8 show that 1 cc. of a 0.001 per cent solution of specific polysaccharide was completely decomposed by 0.5 cc. of the filtrate of an autolyzed culture (Preparation 3-*b*). On the contrary, 0.5 cc. of the filtrate of a young culture (Preparation 3-*a*) did not affect the specific substance. It is apparent, therefore, that the soluble principle responsible for the decomposition of the specific substance is released only after lysis of the cells. This experiment also seems to justify the assumption that the S III bacillus, when grown on a synthetic medium containing the specific polysaccharide, gives rise to a soluble endocellular enzyme capable of decomposing this substance.

Several enzymes are known to be extremely heat-resistant, most of them, however, are thermolabile and it was interesting to determine

the heat resistance of the active principle responsible for the decomposition of the specific substance in autolytic extracts of the S III bacillus.

Experiment 9. Inactivation of the Specific Enzyme by Heat.—Different lots of Preparation 3-*b* were heated at 50°, 55°, 60°, 65°, 70°, and 75°C. for 10 minutes and added in amounts of 0.1 cc. and 0.5 cc. to 1 cc. of 0.001 per cent solutions of SSS III. The mixtures were incubated for 18 hours at 37.5°C. in the presence of toluene and tested at that time for the presence of the specific polysaccharide by the precipitin reaction with Type III serum (see Table IV).

TABLE IV

Heat Inactivation of the Specific Enzyme

Enzyme heated 10 min. at	Specific precipitin reaction of mixture of enzyme and substrate
°C.	
50	−
55	−
60	+
65	+++
70	+++
75	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.
Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

These results indicate that the soluble principle responsible for the decomposition of the capsular polysaccharide is inactivated after exposure for 10 minutes at 60–65°C.

Experiment 10. Activity of the Enzyme under Aerobic and Anaerobic Conditions.—Two tubes containing 2 cc. of a 0.02 per cent solution of SSS III received 2 cc. of active enzyme Preparation 3-*b*. One of the tubes was incubated at 37.5°C. under aerobic conditions, and the other under anaerobic conditions (Brown jar). After 24 hours incubation, the mixtures containing active enzyme and polysaccharide were tested for the presence of specific substance and it was found that the test had become negative in both tubes.

It has been shown previously that the organism is strictly aerobic and does not decompose the specific polysaccharide under anaerobic conditions. This experiment shows, however, that the soluble prin-

ciple, when extracted from the cells, decomposes the specific substance equally well under both aerobic and anaerobic conditions.

These results also indicate that the soluble principle is not of the nature of an oxidative enzyme, since there was no hydrogen acceptor in the mixture incubated under anaerobic conditions. Preliminary results, which indicate the presence of reducing sugars following the decomposition of the specific substance by the soluble principle, suggest that the action is one of hydrolysis.

It has been shown previously (Experiment 7) that of the polysaccharides thus far tested the specific substance of Type III Pneumococcus

TABLE V

Specificity of the Action of the Enzyme Derived from the S III Bacillus

Origin of the polysaccharide	Concentration of the polysaccharide	Specific precipitin reaction of enzyme substrate mixture incubated at 37°C.	
		Aerobically	Anaerobically
	per cent		
Pneumococcus Type I	0.002	+++	+++
Pneumococcus Type II	0.002	+++	+++
Pneumococcus Type III	0.002	−	−
Friedländer's bacillus Type A	0.002	+++	+++
Friedländer's bacillus Type B	0.002	+++	+++
Friedländer's bacillus Type C	0.002	+++	+++
Hemophilus influenzae Type *a*	0.02	+++	+++
Gum arabic	0.01	+++	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

is the only one to be decomposed by the S III bacillus. The following experiment demonstrates that the same extraordinary specificity applies to the activity of the enzyme in cell-free filtrates of autolyzed cultures.

Experiment 11. Specificity of the Action of Enzyme Extracted from the S III Bacillus.—To different portions of enzyme Preparation 3-*b* were added the specific polysaccharides of Pneumococcus Types I, II, and III, Friedländer bacilli Types A, B, and C (11), *Hemophilus influenzae* Type *a* (12), and also gum arabic (13). The mixtures were incubated aerobically and anaerobically and tested for the presence of the polysaccharide after 1 month incubation at 37.5°C. (see Table V).

The results of Experiment 11 indicate that the soluble principle extracted from the bacterial cells is as specific in its action as is the living organism itself, decomposing only the capsular polysaccharide of Type III Pneumococcus.

Since it was proposed to test the effect of this specific enzyme on the course of pneumococcus infection in experimental animals, it was of interest to establish the influence of normal serum on the rate of decomposition of the capsular polysaccharide by the soluble principle.

Experiment 12. The Influence of Normal Serum on the Rate of Decomposition of the Capsular Polysaccharide by the Specific Enzyme.—To each of three tubes

TABLE VI

Influence of Normal Serum on the Rate of Decomposition of the Capsular Polysaccharide by the Bacterial Enzyme

Incubation period	Bacterial extract + specific substance +		
	Salt solution	Rabbit serum	Beef serum
hrs.			
0	++++	++++	++++
1	+++	+++	+++
2	+	++	+
3	±	+	±
4	−	±	−
5	−	−	−

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

containing 3 cc. of enzyme Preparation 3-*b* and 3 cc. of 0.002 per cent solution of SSS III, were added respectively 2 cc. of normal rabbit serum, 2 cc. of normal beef serum, and 2 cc. of physiological salt solution, the last serving as control. The tubes were incubated at 37.5°C. and samples of the mixtures were removed from time to time to follow the progress of the decomposition of SSS III, as measured by the specific precipitin reaction (see Table VI).

The results of this experiment indicate that the rate of decomposition of the Type III polysaccharide by the specific enzyme is not affected by the presence of normal beef or rabbit serum in the mixture.

It was of interest to devise a method for measuring the concentra-

tion or at least the comparative activities of different enzyme preparations. One possible method was to compare the rate of decomposition of a given concentration of specific substance by equal amounts of different preparations. A second method was to determine whether the minimum amount of bacterial enzyme capable of decomposing a given amount of specific substance—independently of time—would vary from one preparation to another, in other words, whether there existed a definite quantitative relationship between total amount of substrate decomposed and amount of active enzyme used. The following experiment was planned to compare the rate of decomposition of the same amount of specific substance by two different enzyme preparations.

TABLE VII

The Rate of Decomposition of Specific Substrate by Two Different Enzyme Preparations

Incubation period	Specific precipitin reaction of enzyme-substrate mixture	
	Prep. 3-*b*	Prep. 4-*a*
hrs.		
1	++++	++
2	+++	++
3	++	−
4	+	−
5	−	−

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

Experiment 13. Comparison of the Activity of Different Enzyme Preparations as Measured by the Rate of Decomposition of Capsular Polysaccharide.—Two preparations (3-*b* and 4-*a*) were compared. The former was the filtrate obtained from an autolyzed culture grown in a mineral medium containing 0.01 per cent of SSS III; 4-*a* was the filtrate of a culture grown in 0.2 per cent SSS III. These two preparations were added in amounts of 5 cc. to 5 cc. of a 0.002 per cent SSS III in buffer pH 7.5. The mixtures were incubated at 37°C. with toluene, and precipitin tests were made from time to time to follow the disappearance of the SSS III (see Table VII).

Table VII shows that when the time required for the decomposition of 1 cc. of a 0.002 per cent solution of specific polysaccharide by 1 cc.

TABLE VIII

Quantitative Relationship between Total Amount of Specific Polysaccharide Decomposed and Amount of Enzyme Used

Enzyme preparation		Specific precipitin reaction of enzyme-substrate mixture incubated for			
No.	Amount	1 hr.	6 hrs.	12 hrs.	24 hrs.
	cc.				
1	2.0	+++	+	–	–
	1.0	+++	++	–	–
	0.5	++++	++++	++	++
	0.2	++++	++++	+++	+++
	0.1	++++	++++	++++	++++
	0.05	++++	++++	++++	++++
	0.02	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
3-*b*	2.0	++	–	–	–
	1.0	+++	+	–	–
	0.5	++++	++	–	–
	0.2	++++	++	+	+
	0.1	++++	++++	++++	++
	0.05	++++	++++	++++	++++
	0.02	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
4-*a*	2.0	+	–	–	–
	1.0	+++	–	–	–
	0.5	+++	–	–	–
	0.2	++++	++	–	–
	0.1	++++	++	–	–
	0.05	++++	++++	+	+
	0.02	++++	++++	++	++
	0.01	++++	++++	+++	++++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

of the bacterial extract is used as a measure of the activity, enzyme Preparation 4-*a* is much more active than Preparation 3-*b*. This

method of titration makes it possible to compare the relative activity of different enzyme preparations.

The following experiment was planned to compare the total amount of specific substance decomposed by given amounts of different enzyme preparations after different incubation periods.

Experiment 14. Titration of Activity of Different Enzyme Preparations as Measured by the Total Amount of Specific Substrate Decomposed.—Three preparations were compared. Preparation 1 was the filtrated autolysate of a culture grown in mineral medium containing 0.001 per cent SSS III; Preparations 3-*b* and 4-*a* were the same as described in the preceding experiment.

The enzyme preparations were added in amounts of 1 cc., 0.5, 0.2, 0.1, 0.05, 0.02, 0.01 cc. to 2 cc. of a 0.001 per cent solution of SSS III; the mixtures were made up to a volume of 3 cc. and were incubated with toluene at 37.5°C. 0.5 cc. samples were taken out from time to time to test for the disappearance of SSS III as determined by the precipitin reaction in Type III antipneumococcus serum.

The results recorded in Table VIII indicate that with a given amount of enzyme the maximum amount of decomposition of specific polysaccharide is reached after 12 hours incubation at 37.5°C. and that no further decomposition takes place thereafter. It appears in this particular instance that it required more than 1 cc. of Preparation 1 to decompose 2 cc. of the standard solution of Type III polysaccharide, whereas the same result was obtained with little more than 0.2 cc. of Preparation 3-*b* and 0.05 cc. of Preparation 4-*a*.

These results indicate that after a definite incubation period, the total amount of specific substrate decomposed bears a quantitative relationship to the concentration and activity of the enzyme preparation used. As a result of these experiments, it has been found that a convenient method of titrating the activity of an enzyme preparation is to determine the minimum amount that would decompose 1 cc. of a 0.001 per cent solution of specific capsular polysaccharide in 18 hours at 37.5°C.

DISCUSSION

The technique of isolation, and the biological characteristics of the bacillus described in this paper illustrate the great possibilities of the "starvation" method for stimulating certain potential properties of microorganisms. Although limited in its ability to utilize carbohy-

drate other than the capsular polysaccharide of Type III Pneumococcus, this organism grows rapidly and abundantly on ordinary media, plain broth, peptone solution, and casein hydrolysate. When grown on these media, however, the presence of other more readily available nutrients exerts a sparing action on the capsular polysaccharide.

When first isolated, the organism required 10 days to decompose the specific substance in concentration of 0.002 per cent; in its present state of activity, it decomposes a 0.01 per cent concentration of the polysaccharide in 24 hours. By repeated transfers in the specific medium this potential property has been greatly enhanced. It seems likely that this increase in activity is associated with an increased elaboration of the specific enzyme. Further data concerning this point will be presented later.

The fact that the growth of this organism is at least partly inhibited by the presence of glucose in the medium is unexplained but not a completely new phenomenon. There are on record at least two cellulose-decomposing species of bacteria the growth of which is inhibited by reducing sugars and especially glucose. It may be mentioned in passing that these organisms (*Sp. cytophaga* Hutchinson and Clayton (14), and "Y" bacillus Dubos (4)) are also extremely specific in their activities since cellulose is the only material on which it has been possible to grow them.

A comparison of the heat resistance of the spores and of the active enzyme extracted from the bacterial cells brings out the interesting fact that whereas the former resist heating at 95°C. for 5 minutes the latter is inactivated by 10 minutes exposure to 60°C.

It is also worth noting that although the organism is so strictly aerobic, the isolated enzyme responsible for the decomposition of the specific substance is equally active under anaerobic and aerobic conditions. It is likely that this soluble principle belongs to the group of hydrolytic enzymes.

Further work is in progress concerning the cultural conditions affecting the elaboration and activity of the enzyme. A fact of practical importance for experimentation is the possibility of titrating the activity of the enzyme *in vitro* by taking advantage of the quantitative relationship which exists between the total quantity of substrate decomposed and the amount of enzyme used.

Finally it may be mentioned that the first question which fostered this inquiry has been answered. The decomposition of the capsular polysaccharide of one of the specific types of Pneumococcus by a mild enzymatic action results in the loss of specific precipitability of this substance in antipneumococcus serum of the homologous type; that the polysaccharide, and not some impurity carried along with it, is responsible for type specificity, is once more proved, and probably beyond doubt.

The specificity of the types of Pneumococcus is illustrated also by the remarkably specific action of this enzyme which attacks only the capsular polysaccharide of Type III Pneumococcus; in fact this enzyme appears as specific as an antibody.

The answer to the two other questions mentioned at the beginning of this paper, namely the influence of the enzyme on the growth of Type III Pneumococcus *in vitro* and on the course of pneumococcus infection in experimental animals will be considered in subsequent papers.

SUMMARY

1. An organism has been isolated from peat soil which decomposes the specific capsular polysaccharide of Type III Pneumococcus.

2. The isolation has been made possible by the use of a synthetic mineral medium containing the specific polysaccharide as sole source of carbon. By repeated transfers in this medium the potential capacity of the organism to decompose the specific substance has been progressively increased.

3. The organism is a pleomorphic bacillus, motile and spore-bearing, exhibiting metachromatic granules; its reaction to the Gram stain varies according to the medium on which it is grown. It is strictly aerobic and grows well in plain broth and peptone solutions; it does not produce gas in any media and it forms small amounts of acid only on dextrin, galactose, lactose, salicin, and trehalose; its growth is inhibited by glucose.

4. The organism decomposes the capsular polysaccharide of Type III Pneumococcus aerobically, between pH 6.2 and 7.8, at room temperature and at 37.5°C., but not at 54°C. The decomposition of the specific substance is inhibited by the presence in the medium of other

nutrients, such as peptones, which act as a more readily available source of energy. The action of the organism is specific; it does not attack the soluble specific substance of Type I or Type II Pneumococcus, nor any of the other bacterial polysaccharides thus far tested.

5. The organism possesses an endocellular enzyme. This enzyme has been extracted by autolysis of the bacterial cells; in sterile solution it exhibits the same specific action as do the organisms from which it is derived, decomposing only the capsular polysaccharide of Type III Pneumococcus.

6. This enzyme decomposes the Type III specific polysaccharide under anaerobic as well as under aerobic conditions; it is inactivated at 60–65°C.; the rate of decomposition of the specific substance is not affected by the presence of normal serum.

7. There exists a quantitative relationship between the total amount of specific substance decomposed and the amount of enzyme preparation used; the existence of this relation makes it possible to express the activity of a given enzyme preparation in terms of the minimal amount required for the complete decomposition of a given amount of specific substance.

8. The specific decomposition of the capsular polysaccharide of Type III Pneumococcus, by the organism as well as by the enzyme it produces, illustrates once more the specificity of the types of Pneumococcus and confirms the fact that the capsular polysaccharides, and not some impurities carried along with them, are responsible for type specificity.

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THE PROTECTIVE ACTION OF A SPECIFIC ENZYME AGAINST TYPE III PNEUMOCOCCUS INFECTION IN MICE

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Plate 4

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The cultural characteristics of a bacillus capable of decomposing the capsular polysaccharide of Type III Pneumococcus were described in the preceding paper (1). The decomposition of the specific carbohydrate was shown to be due to the action of an intracellular enzyme which may be extracted in active form from the bacilli. It was pointed out that the enzyme acts only on the capsular polysaccharide of Type III pneumococci and does not affect the specific carbohydrates of certain other encapsulated bacteria. In this respect, the enzyme exhibits a selective action which is as unique in its specificity as is the serological reaction between the capsular polysaccharide and homologous antibody. Furthermore, it was shown that the breaking down of the complex sugar by the enzyme is accompanied by a loss of the serological specificity of the carbohydrate. This form of decomposition by a biological agent represents a splitting which is less drastic than that accomplished by chemical hydrolysis with acid and heat. The fact that the breaking down of the sugar molecule is, in each instance, accompanied by the loss of its immunological specificity furnishes convincing evidence that the capsular polysaccharide itself is the substance responsible for the type specificity of pneumococci.

The observations recorded in the preceding paper concerned the action of the enzyme on the chemically purified polysaccharide removed and separate from the bacterial cells. In its native state the capsular substance forms a morphological structure which conditions the antigenic and serological reactions of the cell as a whole, as well as its power to invade and multiply in the animal body. It was of special interest, therefore, to ascertain what effect this specific enzyme

would have upon the encapsulated cells growing *in vitro* and *in vivo*; whether in a medium containing the active enzyme Type III pneumococci would fail to grow, or would grow merely deprived of their capsules; whether in the body of a susceptible animal the administration of the enzyme would in any way modify the course of experimental infection with virulent Type III pneumococci. It is with these two questions that the present paper deals.

Before discussing the experimental results to be reported in this paper, it may be well at this point to mention briefly the observations of other investigators who have attempted to modify or inhibit the activity of bacteria by the use of certain enzymes.

Reference has already been made in previous papers (1–3), to the work of Toenniessen (4) who isolated in the form of galactan a gum-like material from an encapsulated strain of Friedländer's bacillus. Of special interest in this regard is his observation on the utilization of this capsular substance by *Bacillus vulgatus*, when both organisms were grown together in symbiosis. Under these conditions he observed that the Friedländer bacilli progressively lost their capsules, although the viability of the decapsulated forms was in no way impaired. This investigator attached no immunological significance to the capsular carbohydrate, nor did he attempt to apply the principle involved in this symbiotic relationship to the problems of experimental infection. Rather, Toenniessen regarded the substance of the capsule as a reserve food stuff, similar to starch in plants and glycogen in animals. From this viewpoint, however, he emphasized the significance of the capsule as a physiologically important part of the bacterial cell and showed that it may be decomposed by an unrelated species of microorganism.

In 1910 Vaudremer (5) pointed out that tuberculin, when added to filtered extracts of *Aspergillus fumigatus*, loses in great part its activity. This decomposition he attributed to the presence of various enzymes in the unheated extracts. He also showed that tubercle bacilli were modified by maceration in extracts of the fungus, and that under these conditions they became granular and less acid-fast. The injection of the bacilli, altered by growth in the fungus extracts, served to stimulate in animals an increased resistance to subsequent infection with virulent tubercle bacilli. Filtered extracts of the *Aspergillus* alone were used in the treatment of guinea pigs previously infected with tubercle bacilli. In these instances, the progress of the disease was often much retarded and the life of the treated animals prolonged. When Vaudremer had shown that unheated extracts of *Aspergillus fumigatus* were able to destroy tuberculin, to diminish the acid resistance of tubercle bacilli, and to retard infection in experimental animals, he applied the same methods to the treatment of tuberculosis in man.

That tubercle bacilli lose their acid fastness after 3 months incubation in a

culture of an unidentified mold was also observed by Machado (6) who traced this action to the activity of the fungus enzymes.

The suggestion that the lipoid substances of tubercle and lepra bacilli may be acted upon by the esterases of the tissues is found in the observations of Citron and Reichen, Peritz and others (7), who have expressed the view that an increased content of these enzymes in the serum of patients is associated with an increased resistance to infection. The relative resistance of the skin to tuberculosis, as Porter pointed out, may possibly be related to the presence of the tissue lipase, although as Sexsmith and Petersen (8) suggest this resistance cannot be due wholly to the activity of this ferment since lepra bacilli, also rich in lipoids, invade the skin. The observation by Robinovitch, Stiles, and Payne (9) that tuberculosis of the pancreas is relatively uncommon in tuberculous animals led these workers to attempt to influence the disease in experimentally infected guinea pigs by the injection of pancreatic extracts containing active lipase.

The active principle, pyocyanase, present in autolysed cultures of *B. pyocyaneus* was found by Emmerich and his associates (10) to bring about rapid dissolution of many different species of living bacteria. They considered the active agent to be an enzyme belonging to the class of nucleases. Active preparations of pyocyanase were used in the treatment of animals experimentally infected with anthrax bacilli. The protection afforded by pyocyanase was attributed to the direct action of the enzyme on the infecting bacilli.

EXPERIMENTAL

Culture.—Young, plain broth cultures of a strain of Type III Pneumococcus were used in the following experiments. The virulence of the strain, maintained by repeated mouse passage, was such that 0.00000001 cc. of culture injected intraperitoneally into white mice caused death within 36 to 72 hours. The dilutions of the culture were made in broth in such manner that the infecting dose was always contained in a constant volume of 0.5 cc.

Enzyme.—Filtered solutions of the enzyme extracted from the S III bacillus were prepared as described in the preceding paper.

Protection Tests.—White mice, 18 to 20 gm. in weight, served as test animals. All injections were made intraperitoneally and the technique commonly used in protection tests with antipneumococcus serum was employed. Unless otherwise stated, the infecting organisms and enzyme were injected simultaneously. In all instances the virulence of the culture was controlled by injecting normal mice with minimal amounts of the culture alone as shown in the protocols.

I. Action of the Enzyme on Encapsulated Type III Pneumococci in Vitro

Reference has already been made to the observations of Toenniessen (4) on the disappearance of the capsules of Friedländer bacilli when

these organisms are grown in symbiosis with *B. vulgatus*. Similar relationships have been found to exist in the case of the symbiotic growth of an encapsulated strain of Type III Pneumococcus together with the enzyme-producing bacillus. The specific enzyme elaborated by the bacilli brings about the decomposition of the capsular polysaccharide formed by the Pneumococcus. In symbiotic cultures of these two organisms, the pneumococci lose their agglutinability in type-specific serum, and the soluble specific substance can no longer be demonstrated free in the culture fluid. Obviously these results are conditioned by certain variable factors, such as the relationship between the rate of decomposition and the rate of production of capsular substance, and the maintenance of cultural conditions suitable for the physiological activity of both species of microorganisms. However, there is no difficulty in demonstrating the reaction when, instead of the living bacilli, a sterile extract of the enzyme itself is added in suitable concentration to the culture medium. Under these conditions the decomposition of the capsular polysaccharide by the enzyme can be demonstrated during growth of the pneumococci, by failure of the culture to react specifically when added to Type III antipneumococcus serum.

Experiments of this nature reveal the fact that the enzyme by itself is neither bactericidal nor bacteriolytic; that by decomposing the specific carbohydrate, the enzyme merely deprives the bacteria of their capsules without impairing the viability of the cells. It is also evident that the action of the enzyme does not result in a loss of the function of elaborating the capsular substance, since organisms so treated regain their capsule and form the specific polysaccharide when transferred to a medium free of the enzyme.

The experiments reported in the preceding paper (1) showed that an enzyme derived from an unrelated species of microorganisms acts upon the Type III capsular polysaccharide of Pneumococcus when this substance, separated from the bacterial cells, is used as the specific substrate. The experiments just described afford evidence that the enzyme also brings about the decomposition of this same capsular material in the native form in which it exists as a structural part of the living organism. Since it has been shown that the enzyme has a definite effect upon the capsular component of the cell growing *in*

vitro, the question naturally arose, whether it would exert a similar action on the encapsulated forms growing in the animal body; to seek the answer to this question the following experiments were designed.

II. The Action of the Enzyme on Encapsulated Type III Pneumococci in Vivo

Specificity of the Action of Enzyme in Mice.—To determine whether the enzyme would protect mice against infection, and to what extent the specificity exhibited by the enzyme *in vitro* would be reflected in

TABLE I

Specificity of the Protective Action of Enzyme

Infecting dose of Pneumococcus	Enzyme (Lot 4-*a*) 0.5 cc.			No enzyme		
				Virulence controls		
	Pneumococcus Type I	Pneumococcus Type II	Pneumococcus Type III	Type I	Type II	Type III
cc.						
0.1	—	—	S	—	—	—
0.01	—	—	S	—	—	—
0.001	—	—	S	—	—	—
0.0001	D 20	D 34	S	—	—	—
0.00001	D 24	D 34	S	D 22	D 36	D 34
0.000001	D 34	D 34	S	D 34	D 36	D 34
0.0000001	—	—	—	D 34	D 20	D 72

S = survived.

D = death of animal; the numeral indicates the number of hours before death, or the time at which the animal was found dead.

— = not done.

its action in animals, three separate groups of mice were infected with Pneumococcus Types I, II, and III, each animal receiving at the same time a constant amount of the Type III specific enzyme. The experiment was carried out as follows:

The technique of the protection test was the same as that already described. Three mice were injected intraperitoneally with 10^{-4}, 10^{-5}, and 10^{-6} cc. of a broth culture of Type I Pneumococcus; three other mice received the same amounts of a culture of Type II; six mice were given much larger doses of a culture of Type III pneumococci ranging in amounts from 10^{-1} to 10^{-6} cc. Simultaneously with the bacteria each mouse of all three groups received 0.5 cc. of the same preparation of

enzyme (Lot 4-*a*). The virulence of the three types of pneumococci was controlled in each instance by the infection of normal mice with 10^{-5}, 10^{-6}, and 10^{-7} cc. of the respective culture alone.

The results of this experiment are recorded in Table I. The evidence clearly shows that the enzyme afforded mice protection against 1 million times the fatal dose of a virulent culture of Type III Pneumococcus. It is further apparent that the protective action is type-specific, since mice receiving the same enzyme but infected with amounts as small as 0.000001 cc. of a culture of a heterologous type promptly succumbed to infection. Just as in the test tube, the enzyme acts only on the Type III polysaccharide, so in the animal body it is effective only against infection with Type III Pneumococcus.

Heat Inactivation of the Enzyme.—Experiments previously reported (1) showed that the activity of the enzyme, as measured by the decomposition of the free carbohydrate, was destroyed by exposure to a temperature of 60°C. for 10 minutes. In the following experiment, an active preparation of the enzyme was heated, at a temperature known to destroy completely its action in the test tube, in order to determine the effect of heat upon the protective power of enzyme in mice.

5 cc. of enzyme preparation (Lot 4-*a*) were heated in a water bath at 70°C. for 10 minutes. Each of three mice was injected intraperitoneally with 0.5 cc. of the *heated* enzyme together with a culture of Type III Pneumococcus in amounts of 10^{-4}, 10^{-5}, and 10^{-6} cc. respectively. Each of five other mice received similar amounts of the same preparation of the *unheated* enzyme simultaneously with an amount of the cultures varying from 10^{-1} to 10^{-5} cc., respectively. Three mice infected with culture alone in doses of 10^{-5}, 10^{-6}, 10^{-7} cc. were used as virulence controls.

The results of this experiment show that the active principle responsible for the protection of mice against infection is destroyed by exposure to 70°C. for 10 minutes. Mice receiving the *heated* enzyme succumbed to infection with the smallest dose of culture used, 0.000001 cc. On the other hand, mice injected with an equal amount of the same enzyme *unheated* survived as much as 0.1 cc. of the same culture of which 0.0000001 cc. alone proved fatal for the untreated controls. The inactivation by heat of the protective power of the enzyme in the animal body, parallels the loss of its activity *in vitro* after exposure to

temperatures of 60°C. or higher. The fact that heat destroys the activity of the enzyme both *in vitro* and *in vivo*, supports the assumption that the same principle is involved in the mechanism of both reactions.

Relation between the Concentration and Protective Action of Enzyme.—Repeated tests have demonstrated that within the limits of the reaction capacity of the mouse, the protective action of the enzyme is a function of the concentration of the active principle in any given preparation. It is also apparent that the protection afforded by different

TABLE II

Inactivation of Enzyme by Heat

Pneumococcus Type III	Enzyme (Lot 4-*a*) 0.5 cc.		No enzyme
	Unheated	Heated at 70°C. 10 min.	Virulence control
cc.			
0.1	Survived	—	
0.01	Survived	—	
0.001	Survived	—	
0.0001	Survived	D 34	
0.00001	Survived	D 72	D 34
0.000001	—	D 72	D 34
0.0000001*	—	—	D 72

D = death of animal; the numeral indicates the number of hours before death.
— = not done.
* The inoculum at this dilution of culture (10^{-7} cc.) yielded 13 colonies on growth in blood agar.

preparations of the enzyme bears a relation to their capacity to decompose a known quantity of the capsular polysaccharide *in vitro*.

The comparative protective action in mice of two separate preparations, one of which was much less active *in vitro* is shown in Table III.

From Table III it is evident that Preparation 3, which was much less active than Preparation 4-*a* in decomposing the polysaccharide in the test tube, is also the less effective in the animal body. Although the results obtained by the two methods of titration are not wholly comparable, they indicate the existence of a correlation between the *in vitro* and *in vivo* activity of the enzyme. The comparison of these two

different preparations also suggests that the concentration of the enzyme in Preparation 3 is close to the minimum threshold value below which the enzyme ceases to function in protecting against even minimal infecting doses. From this and other similar experiments, the impression is gained that to be effective in animal protection, the concentration of enzyme in the body must be in excess so that the rate of decomposition is greater than the rate of production of the capsular substance by the living bacteria. The evidence also indicates that the greater the activity of the enzyme *in vitro* the greater is its protective

TABLE III

Protective Action of Enzyme against Infection with Pneumococcus Type III

Difference in protection titre of two preparations of enzyme which show marked differences in their activity *in vitro*.

Pneumococcus Type III	Enzyme 0.5 cc.		No enzyme
	Lot 3	Lot 4-*a*	Virulence controls
cc.			
0.1	—	S	
0.01	D 45	S	
0.001	D 72	S	
0.0001	D 72	S	
0.00001	S 6 days	S	D 34
0.000001	S	S	D 34
0.0000001	—	—	D 72

S = survival of animal.
D = death of animal; the numeral indicates the number of hours before death.
— = not done.

action in mice. Both of these deductions, however, suffer the limitations imposed by the variations which occur in the cellular response of the host, as will be pointed out later in discussing the importance of phagocytosis in the protective reaction.

Titration of Protective Action.—The concentration of enzyme in any given preparation as measured by the protection test in mice may be ascertained in two ways; (1) by determining the protective action of varying amounts of enzyme against a fixed quantity of culture, and (2) by determining the maximum amount of culture against which a constant quantity of the enzyme will protect. The results of experi-

ments of this nature are given in Tables IV and V. Although in the present instance both methods of titration were not carried out on the same preparation, the examples given illustrate the general principle.

Table IV shows that 0.1 cc. of enzyme preparation protected mice against 0.01 cc. of a virulent culture, an amount 1 million times greater than the minimal dose fatal for the normal controls.

TABLE IV

Titration of Protective Action of Enzyme against Infection with Pneumococcus Type III

1. Varying amounts of enzyme and constant amount of culture.

Enzyme (Lot 7)	Pneumococcus Type III (1 million fatal doses)		Virulence controls
Amount	Amount	Result	
cc.	*cc.*		
1.0	0.01	S	
1.0	0.01	S	
0.5	0.01	S	
0.5	0.01	S	
0.25	0.01	S	
0.25	0.01	S	
0.1	0.01	S	
0.1	0.01	S	
0	0.000001 cc.		D 45
0	0.0000001 cc.*		D 35
0	0.00000001 cc.†		D 46

S = survival of animal. Observation period 10 days.

D = death of animal; the numeral indicates the number of hours before death of animal.

* Inoculum of this amount of culture (10^{-7} cc.) yielded 30 colonies in blood agar.

† Inoculum of this amount of culture (10^{-8} cc.) yielded 1 colony in blood agar.

The results presented in Table V illustrate the maximum amount of culture against which 1 cc. of enzyme preparation (4-*a*) protected mice. Under the experimental conditions, this particular preparation protected against infection with 0.2 cc. of culture, an amount which represents 2 million fatal doses. Death was delayed in mice infected with 0.3 and 0.4 cc. of culture, the animals surviving as long as 4 days. Large infecting doses of this order however apparently represent the

upper limit against which a single dose of the enzyme fails to protect. Whether repeated injections of enzyme during the course of the infection, or whether the perfecting of methods for concentrating and purifying the enzyme will enhance its protective action, cannot now be stated. Moreover the maintenance of an effective concentration of enzyme in the body during the course of infection appears necessary to achieve the maximum of protection.

In order to ascertain how long the enzyme remains active after injection into normal mice, the following experiment was carried out.

TABLE V

Titration of Protective Action of Enzyme, against Infection with Pneumococcus Type III

2. Constant amount of enzyme and varying amounts of culture.

Pneumococcus Type III	Enzyme (Lot 4-*a*)	
	Amount	Result
cc.	*cc.*	
0.5	1.0	D 18
0.4	1.0	D 4 days
0.3	1.0	D 4 days
0.2	1.0	S
0.1	1.0	S
0.01	1.0	S
0.000001	0	D 25
0.0000001	0	D 42

D = death of animal; numeral indicates number of hours before death.
S = survival of animal.

Mice were injected intraperitoneally with 1 cc. of a sterile solution of enzyme. At intervals thereafter varying from 20 to 43 hours, the mice were infected by the intraperitoneal injection of varying amounts of a virulent culture of Type III Pneumococcus.

The results given in Tables VI and VII show that there is a gradual diminution in the protective action of the enzyme after its injection into the animal body. To determine more accurately the length of time during which the enzyme remains active in normal animals will require further detailed study. From the evidence available at present, it seems not unlikely that the occasional death of an infected

TABLE VI

Protective Action of Enzyme against Infection with Pneumococcus Type III

Action of enzyme given 24 hours before and simultaneously with the infecting dose of culture.

Pneumococcus Type III	Enzyme (Lot 7) 1 cc.		No enzyme
	Simultaneously	24 hrs. before	Virulence controls
cc.			
0.2	S	D 6 days	
0.1	S	S	
0.01	S	S	
0.001	S	S	
0.0001	S	S	
0.000001	—	—	D 45
0.0000001	—	—	D 35
0.00000001	—	—	D 45

D = death of animal; the numeral indicates the number of hours before death.
S = survival of animal—observation period 10 days.
— = not done.

TABLE VII

Protective Action of Enzyme Given before Infection with Pneumococcus Type III

Action of enzyme given 20 to 43 hours before infection.

Pneumococcus Type III	Enzyme (Lot 6) 1 cc.	Enzyme (Lot 4-*a*) 1 cc. given		Virulence controls
	20 hrs. before infection	24 hrs. before infection	43 hrs. before infection	Culture alone
cc.				
0.1	S	D 24	D 48	—
0.01	S	S	S	—
0.001	S	S	S	—
0.0001	S	S	D 8 days	—
0.00001	S	S	S	—
0.000001	S	S	S	D 46
0.0000001	—	—	—	D 46
0.00000001	—	—	—	D 46

S = survival of animal.
D = death of animal; the numeral indicates the number of hours before death.
— = not done.

animal several days after the administration of a single dose of the enzyme (Table VII) may be attributable to the loss or inactivation of the active agent during the course of the infection, thereby allowing a few organisms to escape and reestablish themselves in the absence of an effective concentration of the protective principle.

Curative Action of the Enzyme in Mice.—The preceding experiments were designed to demonstrate the protective action of the enzyme when administered simultaneously with the infecting microorganisms. In a few experiments the enzyme was given several hours before the bacteria were injected, in order to ascertain the duration of the activity of enzyme in the animal body. The degree of protection afforded under these circumstances is noteworthy, when the susceptibility of mice to pneumococcus infection, and the virulence of the strain employed are taken into consideration. It seemed of even greater interest, however, to determine whether the enzyme would have any effect when injected into mice that had been previously infected with a fatal dose of culture. The following experiment illustrates the so called curative action of the enzyme in the presence of an infection already established at the time of treatment.

Nine normal mice were infected with 10^{-6} cc. of broth culture of a virulent strain of Pneumococcus Type III. 12 hours later three of these animals were given an intraperitoneal injection of 1 cc. of enzyme (Preparation 4-*a*). Three other mice of the infected group were treated with the same amount of enzyme 18 hours after the infecting organisms had been given. The remaining three mice received no enzyme and served as untreated controls. As further controls of virulence two normal mice were injected with the culture alone in amounts of 10^{-7} and 10^{-8} cc. respectively. To estimate the number of organisms, these inocula were plated in blood agar and the number of colonies developing were counted. 10^{-7} cc. of culture yielded twenty-five colonies in the poured plates, and 10^{-8} cc., three colonies.

The results of this experiment on the curative action of the enzyme (Table VIII) show that mice receiving a single injection of the enzyme 18 hours after the onset of infection, recovered, whereas the untreated controls all died. Under the experimental conditions, the survival of the treated animals represents recovery from an infecting dose of culture 100 times greater than that fatal for untreated mice. While the conditions of this test were not the most severe, the outcome indi-

cates that the enzyme is effective when administered early in the course of an infection which otherwise invariably proves fatal. From other experiments, evidence has been gained that the administration of the enzyme several hours after infection with larger amounts of culture may favorably influence the course of the infection in mice.

Mechanism of the Protective Action.—It was thought possible that some idea of the mechanism involved in the protective action of the enzyme in infected mice might be gained by following the course of the bacteremia by means of blood cultures, and by a study of the

TABLE VIII

Curative Action of Enzyme in Mice Infected with Pneumococcus Type III

Pneumococcus Type III (100 fatal doses)	Enzyme 1 cc. (Lot 4-*a*) given		Controls
	12 hrs. after infection	18 hrs. after infection	No enzyme
cc.			
0.000001	S	S	D 35
0.000001	S	S	D 59
0.000001	S	S	D 59
0.0000001*	—	—	D 84
0.000000001†	—	—	D 60

D = death of animal; the numeral indicates the number of hours before death.
S = survival of animal.
* = Inoculum of this dilution of culture (10^{-7} cc.) gave 25 colonies in blood agar.
† Inoculum of this dilution of culture (10^{-8} cc.) gave 3 colonies in blood agar.

cellular reactions in the peritoneal exudates of treated and untreated animals. A typical experiment follows:

Twelve mice were injected intraperitoneally with 10^{-2} cc. of a culture of Pneumococcus Type III, the virulence of which was such that 10^{-8} cc. caused death in from 36 to 72 hours. Six of the mice also received at the time of infection 0.5 cc. of an active preparation of the enzyme. The other six infected animals served as untreated controls. At hourly intervals following infection, one mouse of each group was sacrificed and autopsied. Cultures of the heart's blood were made in broth and on blood agar plates. Films of the peritoneal exudates were stained by the Gram method, and examined to compare differences in the morphology of the organism and in the occurrence of phagacytosis in the treated and untreated animals.

The results of blood cultures taken during the course of the infection show that the enzyme has a distinct effect in checking the bacteremia which invariably occurs in infected mice. In both the treated and untreated animals pneumococci are present in the blood stream within the 1st hour following infection. However, the subsequent course of events, with reference to the persistence of bacteremia, differs in the two series of animals. For, while the bacteria progressively increase in the blood of the control mice until death, they invariably diminish in numbers in the circulation of the treated animals until by the 4th or 5th hour they can no longer be demonstrated by culture. The sterilization of the blood under these conditions is objective evidence of the protective action of the enzyme.

The fate of the pneumococci under the influence of the enzyme is strikingly revealed by microscopic study of the peritoneal exudate during the course of the infection. Without giving in detail the results of the serial examinations at hourly intervals, the progress of events is shown in the accompanying photomicrographs which illustrate the differences in the cellular reactions of treated and untreated mice 2 and 4 hours after the injection of 1 million fatal doses of Pneumococcus Type III. Two hours after infection the peritoneal exudate of the untreated control mouse (Fig. 1) shows numerous well encapsulated cocci free in the fluid. In contrast to this, the pneumococci in the exudate of the enzyme-treated animal at this time (Fig. 2) are devoid of capsules and only the naked bacteria are visible, many of which are already engulfed by the polynuclear leucocytes. At the end of 4 hours, the number of encapsulated pneumococci have increased in the peritoneum of the untreated control (Fig. 3), there is no evidence of phagocytosis, and cultures of the heart blood indicate a progressively increasing bacteremia. In the treated mouse at the end of 4 hours (Fig. 4) only an occasional unencapsulated organism is seen outside the accumulated leucocytes and frequently at this time and almost invariably by the 5th hour, pneumococci are no longer demonstrable by blood cultures. These findings have been repeatedly confirmed in a series of similar experiments and they support the view that the protective action of the enzyme lies in its capacity to decompose the capsular polysaccharide of Type III Pneumococcus.

DISCUSSION

The present study emphasizes the importance of the capsule in the biological reactions of the pneumococcus. It is, indeed, a significant fact, that no matter whether one regards this organism from the viewpoint of type specificity, antigenicity, or its capacity to undergo variation, or whether, as in the present instance, one considers the pneumococcus with reference to its virulence and fate in the animal body, the one dominant factor influencing all these phenomena is the function of the cell to elaborate the specific capsular polysaccharide. These relationships, however, are not to be interpreted as meaning that virulence is dependent merely upon differences in the structural morphology of the bacterial cell. For it is a common observation that an encapsulated strain of Pneumococcus may be virulent for one species and not for another. However, it is equally true that the function of elaborating the specific capsular polysaccharide is most highly developed in pneumococci that are best adapted to growth in the animal body. From this point of view, virulence and capsule formation, although not causally related, are at least intimately associated. When the function of forming the capsular substance is suppressed or inhibited, as in the case of the R variants, or when, as in the present instance, although this function is unimpaired the capsule itself is destroyed by an enzyme, the naked bacteria are thereby exposed directly to attack by the phagocytes of the host.

In this sense, the action of the enzyme may be said to result in preparing the encapsulated bacteria for phagocytosis; not, as in the case of antibodies, by specific sensitization, but by the process of decapsulation. In the former instance, the reaction is an immunological one, whereby the capsular material is altered by union with the type-specific antibody; in the latter case, the reaction is a chemical one in which the capsular polysaccharide is actually decomposed by the enzyme. Although the mode of action of both these specific agents is different in each instance, the end result, so far as the fate of the microorganism is concerned, is the same in both cases.

It is of interest that although neither the enzyme nor the specific antibody is by itself bactericidal or bacteriolytic, yet each by reacting specifically with the capsular substance exposes the virulent organisms to the phagocytic action of the body tissues. The enzyme, like the

specific antibody, serves merely to initiate the protective reaction, the completion of which is ultimately dependent for its successful issue upon the effective cellular response of the host.

The present study also suggests that the capsule—long recognized as a defense mechanism on the part of virulent bacteria—is a decisive factor in determining the fate of pneumococci in the animal body, and that this structure is vulnerable to attack by specific agents other than antibodies.

SUMMARY

The bacterial enzyme which decomposes the purified capsular polysaccharide of Type III Pneumococcus *in vitro* also destroys the capsules of the living organisms growing in media and in the animal body.

Potent preparations of this same enzyme protect mice against infection with virulent Type III Pneumococcus. The protective action is type-specific.

The protective activity of the specific enzyme is destroyed by heat (70°C. for 10 minutes).

The enzyme remains in an effective concentration 24 to 48 hours after its injection into normal mice.

The enzyme has been found to exert a favorable influence on the outcome of an infection already established at the time of treatment.

A definite relationship has been found to exist between the activity of the enzyme *in vitro* and its protective power in the animal body.

The mechanism of the protective action is discussed with special reference to the relation between the decapsulation of the bacteria by the enzyme and the phagocytic response of the host.

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EXPLANATION OF PLATE 4

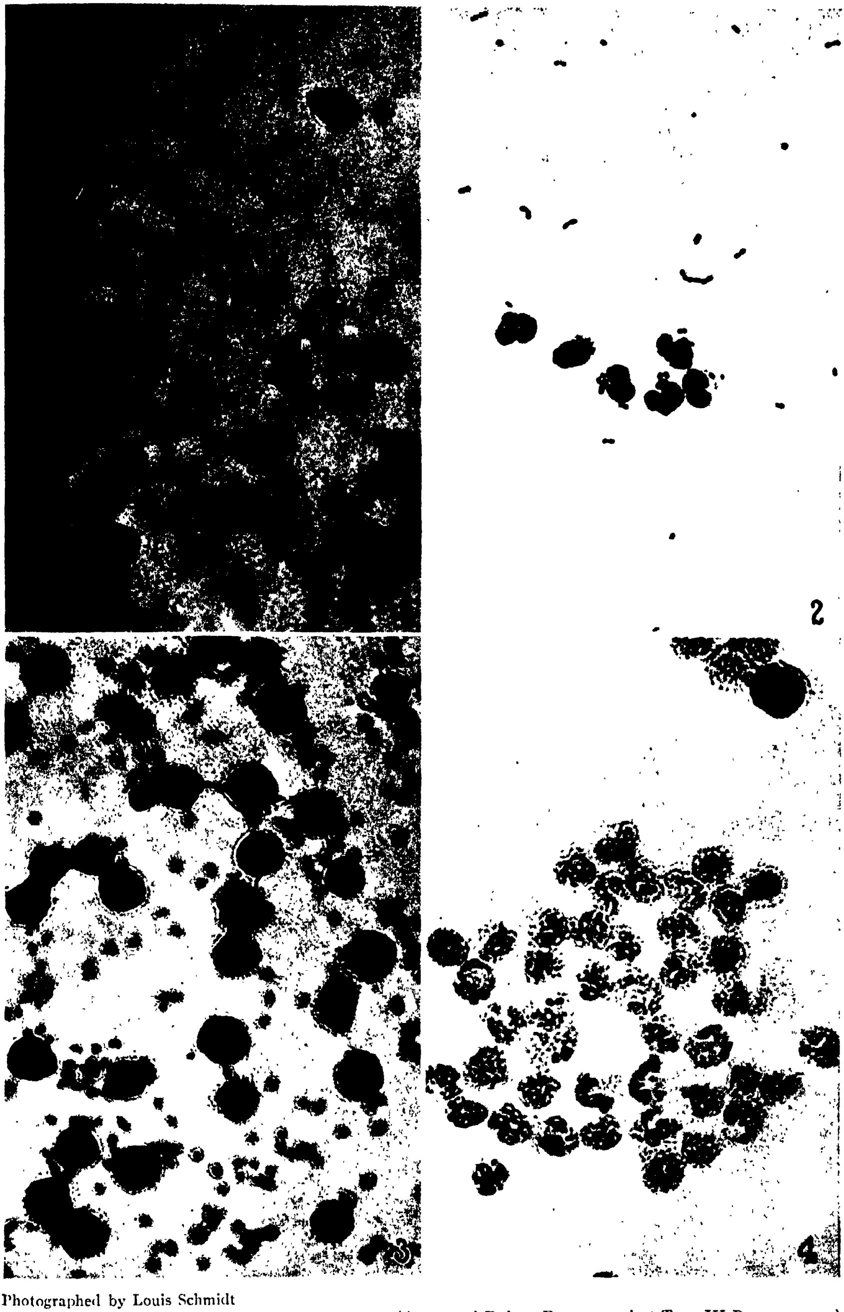
FIG. 1. Photomicrograph of a stained preparation of the peritoneal exudate of a mouse 2 hours after the intraperitoneal injection of 0.01 cc. of a virulent culture of Type III Pneumococcus. The bacteria show well defined capsules and no evidence of phagocytosis is seen. Many polymorphonuclear and a moderate number of mononuclear leucocytes are present. Gram stain, ×1000.

FIG. 2. Photomicrograph of a corresponding preparation of the exudate of a mouse 2 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. The bacteria are devoid of capsules. Polymorphonuclear leucocytes predominate and phagocytosis is evident. Gram stain, ×1000.

FIG. 3. Photomicrograph of a stained film of the peritoneal exudate of a mouse 4 hours after injection with 0.01 cc. of culture alone. The bacteria are increased in number, encapsulated, and extracellular. The cellular elements are polymorphonuclear and mononuclear leucocytes in about equal numbers. Gram stain, ×1000.

FIG. 4. Photomicrograph of a corresponding preparation of the exudate of a mouse 4 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. Marked phagocytosis has occurred and only an occasional organism is seen outside the accumulated leucocytes, nearly all of which are of the polymorphonuclear type. Gram stain, ×1000.

Differences in the density of the backgrounds of the four figures are due to the use of color screens in the photographic reproductions. This technique, however, alters none of the essential details observed in the original microscopic preparations.



Photographed by Louis Schmidt

(Avery and Dubos: Enzyme against Type III Pneumococcus)

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FACTORS FAVORING THE ONSET AND CONTINUATION OF RHEUMATIC FEVER*

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During the past three decades our ideas concerning rheumatic fever have undergone striking evolution, as evidenced by the change in nomenclature from acute articular rheumatism to acute rheumatic fever, and finally to rheumatic fever. True it is that the older terms still hold, and rightly so when used to describe particular forms of the infection; but too often one is employed synonymously with another. It is unfortunate that with increasing knowledge of the condition there cannot be devised a new term sufficiently extensive to embrace all of its manifestations, yet distinctive enough to separate it as a nosological entity. The introduction of the terms "infectious rheumatism"[1] and "rheumatic granulomatosis"[2] are attempts in this direction; but having only pathological or bacteriological significance they offer little if any advantage over the term rheumatic fever, which at least has background in clinical experience.

Objections to the use of this term are twofold: (1) The adjective "rheumatic" to most persons signifies arthritis or muscular pain, and (2) some of the manifestations of activity—notably chorea—are frequently not accompanied by fever. To this one may reply, first, that the original meaning of the term "rheuma" was a morbid process flowing from one organ or tissue to another; hence it still retains its descriptive value; and, second, that regular consistent use of the thermometer would reveal some degree of pyrexia during certain periods of most attacks; therefore, fever still remains one of our most valuable guides of persisting infection.

Another point deserves attention; if the term rheumatic fever is substituted merely for the expression, acute articular rheumatism, our nomenclature has suffered a loss, because the second signifies acutely swollen joints. When, on the other hand, it is used to include all of the manifestations of the infection in the same manner as tuberculosis

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includes all types of the disease induced by the tubercle bacillus, then does it have distinct descriptive value.

To many it seems well to delete the word acute because in the majority of cases the infection is long-standing. Only when rapidly fatal, or in those cases with a monocyclic course may the adjective "acute" be properly applied, but even then we must be certain that the infection has become permanently inactive. For example, we do not picture syphilis as acute even though the roseola disappears within two weeks; likewise it may be better not to use any single manifestation of rheumatic fever as an index of chronicity.

Historically the conception of rheumatic fever began with rheumatic polyarthritis; a century has elapsed since the recognition of the importance of involvement of the valvular endocardium; fifty years ago the nature of the subcutaneous nodule was noted; twenty-five years later its analogue in the submiliary myocardial nodule—the Aschoff body—was described; and within the past decade the extent of the vascular lesions has been appreciated. In the meantime with an improvement in the general economic state of society, together with extensive use of salicylates, the clinical picture has apparently changed. Hyperpyrexia rheumatica has become a medical curiosity, and according to old clinicians rheumatic polyarthritis is less severe. But whether there has been a corresponding amelioration in carditis and chorea one may well doubt. Knowledge gained from contemporary graphic methods of recording cardiac abnormalities renders difficult comparison with statistics obtained in other ways; but figures such as presented by Ehrström and Wahlberg[3] in Helsingfors indicate that there has been no diminution in the incidence of chronic rheumatic heart disease from the administration of salicylates; and according to recent statistics it still seems that rheumatic fever is the largest single factor in the causation of heart disease.[4]

May it not then be of greater value to apply another method of historical approach, beginning in childhood and following the various manners of the unfolding of the infection, rather than to orient ourselves from the disease in adults where it is less frequent even though more acute, and where severe cardiac damage is relatively less common.

But before tracing the clinical course it may be well to reconstruct a background of histopathological tissue changes. What does the

microscope show us concerning the nature of the infection? Aschoff's description of the submiliary nodules arising in the loose connective tissue septa in the myocardium furnished a structural archetype to which alterations in other tissues might be compared. The discussion which has centered about this nodule has at times diverted attention away from the fact that other lesions, possibly not quite so regular in their cellular structure, might be just as characteristic. One need only mention the subcutaneous nodule. The important factors to recall are the type of tissues and organs involved, and the manner of evolution of the lesions; one may then attempt to construct from these factors or elements a hypothesis of the nature of the infection.

In the Aschoff body there is a minute central area of broken collagen fibers, surrounded by large cells probably derived from locally stimulated connective tissue. At times there are seen polymorphonuclear cells, lymphocytes and plasma cells; and finally fibroblasts leading eventually to a scar. The subcutaneous nodule shows qualitatively similar tissue injury and cellular response, but with different quantitative distribution of the component parts; the connective tissue degeneration is often more massive, the groups of large mononuclear cells are more numerous. Frequently a large nodule is apparently formed as a conglomeration of smaller nodules. In places large basophilic mononuclear cells seem to arise from vessel walls. Relatively few polymorphonuclears are present. If now we examine the joints another but related picture appears. The peri-articular tendons and ligaments show numerous microscopic areas in form of nodules or tongues, most having necrotic centers and surrounding proliferated cells. The synovia shows palisading of its lining layer, and minute foci with central necrosis with surrounding collars of cells. Diffuse infiltrations of polymorphonuclears are common. The peri-articular tissues are infiltrated with serum which in part contributes to the familiar swollen joint; and there is destruction of muscle fibers at the musculo-tendinous junctions.[2, 5, 6] In other words, the minute focal and vascular lesions about the joints are numerous, and exudation is widespread. Involvement of the pleura and pericardium shows similar exudative tendency, but in the substance of these membranes are often foci comparable with the Aschoff bodies. In the auricular endocardium there are similar tissue and cellular changes, but these are arranged in streaks and plaques without interruption in the con-

tinuity of the lining endothelium.[7, 8] In the valves on first sight appears another picture, for the endocardium is broken and thrombotic verrucae are often, though not always, laid down at the site of impact of the leaflets. But throughout the substance of the valve and in the chordae may often be seen broken collagen material, proliferative cells and infiltrations like that of the auricular endocardium; at times they are arranged in typical Aschoff bodies. As so beautifully shown by von Glahn and Pappenheimer[9] and others, many portions of the vascular system are similarly involved. In the aorta focal lesions follow branches of the vasa vasorum; but smaller arteries have areas of end- and mesarteritis, always accompanied by focal destruction of connective or elastic tissue. In the peritonsillar, nasopharyngeal and intestinal blood vessels Holsti[10] has demonstrated extensive endarteritis verrucosa. In the peritonsillar capsule near the points of attachment of the pharyngeal muscles MacLachlan and Richey,[11] Gräff[12] and others have described areas very similar to Aschoff bodies, and also in the tongue about the lingual tonsils. Gräff applies the term "primary complex" to these peritonsillar lesions because of their hypothetical rôle as sites whence the infectious agent is distributed to other parts of the body. He thinks that the pathological condition of the blood vessels supplying these lesions favors such a distribution.

The striking picture of the Aschoff body cells has, moreover, attracted attention away from what appears to be the initial injury to the connective tissue fibers. Von Glahn and Pappenheimer have frequently described the granular broken appearance of these fibers, and many have seen the fibrin-like staining in filtration in the foci; but recently Klinge[13] claims that the primary change is a minute focal "fibrinoid swelling" of the intercellular mesenchymal ground substance; which swelling leads secondarily to a fraying out and altered staining reaction of the collagen fibers and fragmentation of the elastica. He also describes waxy degenerations of individual muscle fibers with secondary proliferation of the perimysium.

It thus appears that rheumatic fever instead of affecting any one set of organs is a disease primarily of the connective tissue, or, in Hueck's[14] words, of the mesenchymal system. Those structures composed chiefly of connective tissue, and specially subject to functional stress and strain and undergoing active motion appear to be the most

vulnerable. Interference with the function of these moving structures may, however, detract attention from unobtrusive lesions in other organs, such as recently described by Paul[15] in a rheumatic perihepatitis with characteristic lesions in the underlying blood vessels. Rheumatic vasculitis in the kidneys has been described by Fahr,[16] Evans[17] and others; and symptoms of appendicitis, intimately associated with generalized rheumatic fever, point to a similar involvement of at least one portion of the intestinal tract. A constantly growing literature on pulmonary lesions in this disease indicates also how the lower as well as the upper portion of the respiratory tract may be involved.

A knowledge of the numerous points and tissues where the infectious agent attacks the body gives us another standard with which to judge rheumatic fever. If so many organs or tissues are simultaneously involved, there is every reason to suppose that they may also be individually and successively implicated. In fact, pediatricians have long appreciated the tendency of children to show first one and then another of the so-called rheumatic series;[18] and that not until after the lapse of years might enough members of this series have appeared to render certain a diagnosis. Monosymptomatic signs of disease are difficult of interpretation unless sufficiently characteristic to have diagnostic specificity; for example, the various cutaneous syphilides. But in order to form correct judgment concerning visceral lesions it is often necessary to have concomitant clinical signs or specific laboratory aids.

Unfortunately in the case of rheumatic fever no specific laboratory test is at hand; some of the concomitant, easily visible manifestations, such as tonsillitis, are too nonspecific to furnish much needed assistance. But in these very nonspecific signs we may possess most important aids to understanding the nature of the infection; and in tracing the life history of rheumatic fever it is essential to note their occurrence and then try to interpret their influence upon the course of the malady.

The causation of many chronic diseases is usually the algebraic sum of a number of factors rather than the exclusive action of any one. Infection is the result of interaction between an animal host and an infecting parasite in which many variables are too subtle for laboratory measurement. Moreover, the study of the life history of chronic dis-

ease in the patient gives us many useful hints as to the nature of the illness, and often furnishes therapeutic indications. For example, we now know that the presence of tubercle bacilli in a body does not necessarily indicate active tuberculosis. Certain environmental conditions favor the spread of the lesions; others favor their regression. Indeed, a study of these latter conditions has furnished us with some of our most important weapons against this disease; and comparable knowledge may conceivably have a similar effect in rheumatic fever.

Geographically the disease seems to be essentially one of the temperate zone. Clarke[19] has recently marshalled most convincing evidence indicating that in the true tropics it is fifteen to twenty times less frequent than in Europe. Studies by the Seegals[20] indicate, moreover, that the infection is less common in the southern part of this country; and observations[21] from New Orleans show that when present in the South it runs a milder course than in the North. The ultimate effect of removing rheumatic subjects to hot or dry climates is, however, still a matter for investigation.

Statistics also show that obvious rheumatic fever is from fifteen to twenty times more frequent among the laboring classes than in those forming the bulk of private practice. But many physicians can testify concerning its existence and tendency to progress in patients living under apparently ideal home surroundings. Another viewpoint has been advanced to the effect that among persons in better economic conditions the infection may have relatively more monosymptomatic forms and hence lead more frequently to cardiac damage without obvious general symptoms.

Infants and very young children are relatively free from the disease, and, even though cases appear in children of from two to four years, the curve of frequency of first attacks does not begin its steep ascent until about the age of five or six years. It then rises steadily until the period from nine to eleven years when it begins to fall. First attacks are relatively much more rare in adults than in children. The studies of Wilson, Lingg, and Croxford[22] indicate, moreover, that children suffering from the infection tend to have fewer obvious relapses after the age of eleven or twelve years. Thus a condition of resistance seems to begin to develop about the age of puberty. But the period of greatest incidence of new cases during the first few years of school life

is worthy of emphasis. Is it the result of intimate contact like that seen in measles, or is it due to an age-linked hypersensitiveness? The experience of many observers teaches that the infection is progressing steadily in the hearts of many children while avoiding other organs. For example, Sutton[23] found in the Bellevue Hospital 18 per cent of 427 rheumatic children to have well-developed rheumatic carditis without a previous history of either polyarthritis or chorea.

An apparent precursory factor in a majority of cases is repeated infection in the respiratory tract, often in the form of tonsillitis, sinusitis, middle ear disease, or bronchitis. In our experience so frequent has been the occurrence of acute tonsillitis within from one to five weeks of an acute attack that we now date the duration of a given attack from the onset of tonsillitis. But more detailed investigation of the previous state of health of patients usually reveals an earlier history of repeated sore throats, otitis media, or of recurring or almost continuous sinusitis. Not infrequently closer questioning discloses mild joint or growing pains with these upper respiratory infections. Coates and Thomas,[24] Coates and Coombs,[25] and Vining,[26] all report the finding of small subcutaneous granules in a fairly high percentage of school children. Whether or not these are genuine rheumatic subcutaneous nodules is a moot point; but their alleged demonstration by serious students of the disease should stimulate renewed investigation, because extensive painless nodules have been frequently observed in children having no other symptoms of sufficient severity to incapacitate them.

Other conditions in many children preliminary to an acute attack are loss of weight, anorexia, and general signs of mild intoxication. To these Vining has applied the term "toxic debility," and found that many of his rheumatic youngsters had in addition a history of intestinal disturbance of sufficient severity to point to the intestinal tract as an area whence the infectious agent might be spread throughout the body. The greater liability of children of the poorer classes to suffer disorders of malnutrition or to be deprived of certain accessory food substances suggests that possibly these are elements leading to a higher incidence of the disease among such individuals compared with people living on a higher economic scale. Recent studies of rheumatic children in out-patient departments furnish additional support con-

cerning the influence of nutrition, in that loss of weight has been found to be one of the most common precursors of a relapse.

Recurrences of symptoms sufficiently severe to be called true relapses are commonly observed in children year after year; but symptoms and signs too mild to attract much attention not infrequently occur between relapses. For example, Shapiro[27] has recently noted electrocardiographic evidence of active cardiac damage in at least 60 per cent of 119 school children following apparently complete recovery from an acute attack; and Levy and Turner[28] have recorded electrocardiographic abnormalities weeks and months before the onset of acute symptoms. Persistent low-grade leucocytosis, unexplained on grounds other than that of persisting rheumatic infection, is not infrequent. Several of our patients have observed recurring erythema marginatum for months without appreciating its significance until more incapacitating manifestations forced them to seek hospital care, and the continuance of this peculiar rash for weeks or months following subsidence of acute arthritis not infrequently is an index of continuing infection.

Not only are such pictures seen in children, in whom we have learned to expect repeated relapses, but a similar history is not rare in adults when sought with sufficient care. For example, a man, aged thirty-three years, in good circumstances gave a history of acute tonsillitis in 1929 followed by acute rheumatic polyarthritis, then by tonsillectomy. In the winter of 1930 he had sore throat followed by a similar polyarthritis. But more careful questioning revealed repeated pharyngitis for eight to ten years previously, several attacks of sinusitis and recurring pain in the neck and back of sufficient severity to make movements difficult. It does not appear unreasonable to suppose that during the previous years he was suffering from mild rheumatic infection which was not brought to acute intensity until his first attack of severe tonsillitis. Such histories are not rare.

That tonsillitis plays an important rôle in precipitating many acute attacks of rheumatic fever we can accept as fairly well established. Where both diseases are reportable, the peak of the curve of the former antedates that of the latter by about two weeks. Glover,[29] in presenting details concerning several concomitant epidemics of these two conditions, advances the theory that they are spread by droplet infec-

tion which must reach a certain intensity before the resulting diseases reach epidemic proportions. In one outbreak carefully studied, the carrier rate for meningococci and the incidence of cerebrospinal meningitis and of rheumatic fever ran parallel; and this suggests that similar influences were at play in causing an increase in all three conditions. With a diminution in crowding there was a disappearance of both diseases, and with renewed crowding there was a return of an equal number of each. While these appeared to be primary attacks of rheumatic fever, one would like information concerning the previous history of the rheumatic individuals. Such questions are raised by the study of epidemics among children with rheumatic heart disease or convalescent from rheumatic fever, such as reported by Boas and Schwartz,[30] and Hiller and Graef.[31] In the first epidemic reported by the former group there were four cases of bronchopneumonia accompanied by rheumatic carditis and one case of acute tonsillitis; in the second, although the precursory respiratory infection was not so marked, still acute rheumatic exacerbations developed in six boys in rapid succession. Among 19 nonrheumatic children in the same wards, none suffered from rheumatic fever, while in 22 previously rheumatic subjects 11 developed acute rheumatic fever. In the epidemic reported by Hiller and Graef there were 43 children exposed, of whom 39 probably were previously rheumatic. Within five days of arrival at the camp there were twelve cases of upper respiratory infection, and within five weeks ten cases of polyarthritis, one of chorea, two of bronchopneumonia, one of acute bronchitis, and one of tonsillitis. pericarditis and pneumonia. Unfortunately the exact relationship of the upper respiratory infections to polyarthritis in each case was not recorded; nevertheless the high incidence of acute exacerbations of the disease in previously rheumatic children is worthy of emphasis, as is also the occurrence of severe pulmonary infection.

Scarlet fever is another disease intimately associated both with first attacks and with relapses of rheumatic fever. Some clinicians state that this disease in previously rheumatic children is practically always followed by acute manifestations of rheumatic fever. This suggests the possibility of comparable influences in all of the above mentioned epidemics, namely upper respiratory infection.

I realize fully the possibility of at least two interpretations of the

phenomena described: (1) That rheumatic fever may be due to an unknown virus which may long lie latent in the body, and be incited to renewed activity because of the depressing influence of the acute respiratory infection; (2) on the other hand, it is possible that repeated and persisting low-grade infections induce or are accompanied by tissue changes too mild to be dignified by the name rheumatic fever, and that only with a stormy acute infection such as tonsillitis, or with invasion of the pulmonary tract by streptococci are the accompanying morbid processes raised above the clinical horizon. In either case the conditions existing prior to the attack of acute rheumatic fever are worthy of more detailed study than they usually receive.

To illustrate this point let us consider another group in which a contagious element may exist--the family. Since St. Lawrence's[32] report eight years ago showing the incidence of multiple cases of rheumatic fever in a group of families to be as high as that of tuberculosis, there have been several confirmatory studies. It now appears that in a rheumatic family with one case the probability of occurrence of a second case is three or four times as great as in a family previously free from the disease. Although the various factors favoring the development of rheumatic fever may be almost as difficult of analysis within a family as in any other group, the family as a unit offers a promising field for investigation. For example, several years ago we learned that the mother of one of our rheumatic children frequently had sore throats within a short time of the appearance of relapses in the child. Following the removal of badly diseased tonsils in the mother her sore throat ceased, and since then the child has been free from recurrences. Last winter a boy was admitted to the hospital with the following recent history of acute infections in himself, his mother, and sister:

First day, onset of grippe in patient and sister.

Second day, patient better; sister developed rash.

Eighth day, mother developed grippe with severe pain in back; sister recovered and lost rash.

Twelfth day, mother recovered.

Fourteenth day, patient developed fever and beginning polyarthritis.

Sixteenth day, patient had evidence of severe myocarditis.

Nineteenth day, patient had signs of pericarditis.

Such a history of contagion reminds one of the concomitant respiratory infections in the epidemics mentioned above and suggests the possibility of atypical nondiagnostic manifestations in the sister and mother. Indeed, a correlation of all of the illnesses of the members of fifteen rheumatic families by Paul and Sallinger[33] has already yielded important data along these lines. They have shown that both primary and secondary attacks of rheumatic fever in certain members of a family have been accompanied by the simultaneous appearance of recognizable rheumatic fever in other members of the family, and not infrequently by the appearance of such nonspecific affections as sore throat, bronchitis, bronchopneumonia and skin rashes. They found in addition that the disease spread more frequently to the children under twelve years of age than to the older children and adults. Another striking feature among these families was the frequency with which so-called nonspecific respiratory infections occurred before the appearance of characteristic rheumatic fever. If an extension of this type of investigation yields similar data, we shall be in a position to formulate preventive measures not heretofore applied. Indeed, the information already available from the several reports above reviewed together with those of Andrieu,[34] Grenet,[35] Irvine-Jones[36] and others suggests strongly the communicable nature of the infection. The time when health authorities will recognize this feature of the disease and attempt to assemble data compiled from compulsory notification may be nearer than we can now foresee. The numerical and economic importance of the problem far outweighs that of poliomyelitis, encephalitis, leprosy, and many other reportable diseases.

One more feature deserves attention: the hypersensitiveness of patients with rheumatic fever to streptococcal products, which has been found by most observers to be higher than in any other disease. One must admit that such hypersensitiveness is found in many nonrheumatic persons; hence these skin tests have not diagnostic specificity. But it has offered a possible explanation of certain peculiarities of the disease.[37] Mackenzie and Hanger,[38] Kaiser[39] and Ando[40] have all shown this type of hypersensitiveness to be rare or absent in infancy and to increase in relative frequency with each half decade up to the period of adult life. In Duckett Jones's[41] experience over 95 per cent of rheumatic children gave positive reactions to a filtrate of a single strain of

indifferent streptococci. Derick and Fulton[42] have recently found skin hypersensitivity to hemolytic streptococcal nucleoproteins in 88 per cent of rheumatic children between six and ten years of age compared with only 12 per cent in nonrheumatic children, surely a most significant difference, when it is noted that 88 per cent of their entire group of rheumatic fever patients gave positive reactions. It thus seems that hypersensitiveness to streptococci which appears with advancing years in many individuals occurs much earlier in rheumatic fever patients. Probably the repeated respiratory infections already so frequently mentioned are factors in conditioning a high sensitivity to streptococci; and it does not seem improbable that the condition recognized as acute rheumatic fever is incited by intense, focal infections such as acute tonsillitis, or otitis media—both due to hemolytic streptococci—occurring in already somewhat hypersensitive bodies. On the other hand, it must be recognized that we do not know definitely whether the relationship between streptococcal hypersensitiveness and rheumatic fever is causal or merely concomitant. Its existence, however, gives us a definite point of attack,[43] for could we influence it in one direction or another we would have an index with which to judge the effect of certain therapeutic efforts.

SUMMARY

Rheumatic fever is one of the most important of diseases economically, not only because of its acute manifestations, but also because of its rôle in the production of between 30 and 40 per cent of chronic cardiac disease in the latitude of the North Atlantic States. Microscopic findings indicate it to be a widespread disease involving by preference mesenchymal structures or mesenchymal portions of parenchymatous organs. Physiological stress and strain appear to favor localization of its manifestations, although it may be locally active without giving rise to symptoms; and various vulnerable organs may be either simultaneously or independently involved.

A number of factors appear to have causative relationships. Climatic conditions such as exist in temperate zones in winter favor its development; while sunny dry summers and tropical weather inhibit or prevent its evolution. Among the poorer classes it is from fifteen to twenty times more prevalent than in persons better housed and fed.

Malnutrition and mild toxic states are frequent precursors of characteristic attacks. Most patients, both children and adults, give a history of repeated nonspecific infections of the respiratory tract, tonsils, sinuses, or middle ear, extending over several years, before a typical attack of rheumatic fever is ushered in by a severe focal infection. Persons in intimate contact with patients during acute outbursts of rheumatic fever not infrequently suffer simultaneously from upper respiratory infections, or from typical rheumatic fever; hence there seems to be a distinct communicable factor favoring its spread. Because chronicity and relapses are so frequent, and crippling cardiac damage is of such gradual evolution, and finally because laboratory tests often reveal activity in periods between attacks, it seems justifiable to consider the infection to be characterized by long periods of preparation or sensitization of the tissues, the result of repeated mild infection before a more intense focal infection sets off the violent explosion recognized as acute rheumatic fever. Similar mild infections, moreover, apparently favor the continuation of true rheumatic activity in viscera already involved. It seems logical, therefore, to regard these preparatory periods and mild chronic infections between acute outbreaks as essential parts of the morbid process; hence our therapeutic and prophylactic efforts should be directed against them as well as against the more acute manifestations of the disease.

Note at time of correction of proof: While this article was in press the monograph[44] of Coburn appeared which presents much valuable additional evidence concerning the chronic and contagious nature of rheumatic fever; its geographic distribution; the high degree of cutaneous sensitivity of rheumatic fever patients to hemolytic streptococcal nucleoprotein; and finally a striking parallelism between an increase of hemolytic streptococci in the upper respiratory tract and relapses in patients with the disease. He apparently wishes to designate the malady as the "rheumatic state" and only recognizes "rheumatic fever" when it is diagnostically clear cut.

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PSITTACOSIS

I. EXPERIMENTALLY INDUCED INFECTIONS IN PARROTS

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PLATES 5 TO 7

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The widespread outbreak of psittacosis in man in 1929–30 led to renewed interest in the disease which quickly resulted in the discovery of the facts that the causative agent is not Nocard's bacillus but a filterable virus (1) and that the incidence of laboratory infections is high. Because of these facts it became imperative that further work on the nature of the etiological agent and the mode of spread of the infection be undertaken.

Krumwiede and his coworkers (2) were among the first to show that the inciting agent of psittacosis is capable of passing filters and that mice are susceptible to the disease. Due to his ill health, however, and because of the development of psittacosis in several of his assistants, investigations so admirably begun by him had to be discontinued and two strains of the virus with which he was working were given to us for further study. The purpose of this paper and the three that follow is to present in detail investigations, already described in preliminary papers (3–5), concerning psittacosis experimentally produced in parrots, mice, rabbits, guinea pigs, and monkeys. Special attention has been paid to the mode of spread of the disease and to the pathological changes induced, depending upon the host and upon the portal of entry of the virus.

Methods and Materials

Virus—Krumwiede provided us with two strains of virus, one from a patient, the other from a parrot. Parrot Wenz C after feeding upon lung and spleen from a fatal case of psittacosis was transferred to our laboratory. Parrots N, O, and R were inoculated intramuscularly with spleen emulsion from Parrot M, and Parrot K was infected intramuscularly and intraorally with organ filtrate from Parrot

F. Following inoculation these birds were sent to The Rockefeller Institute. From examination of Text-fig. 1, it will be seen that the virus with which Parrots K, N, O, and R were inoculated came originally from a natural infection in Parrot 17. In Text-fig. 1, the doubly starred birds were handled only by Krumwiede and his coworkers, the singly starred parrots were inoculated by them and given to us, the birds without stars were seen and handled only by us.

Parrots.—Most birds used were Amazon parrots, while the remainder came either from Cuba or Mexico.

Inoculations.—Inoculations were effected by instillation of the virus in the nose and mouth, by pollution of the food and drinking water with the inciting agent, or by injection of the virus into the pectoral muscles.

Examination of Tissues.—The majority of the autopsies was performed immediately after the animals had died or after they had been sacrificed by means of chloroform. In all instances, aerobic and anaerobic cultures from various organs were made for the detection of ordinary bacteria. Sections from tissues fixed in Zenker's fluid and in 10 per cent formalin were stained with eosin and methylene blue or according to Wolbach's modification of Giemsa's method.

Measures Employed to Prevent Laboratory Infections.—At the time we began our work it had already become apparent that laboratory infections are a menace. Consequently measures were instituted to prevent as far as possible the spread of the disease to those actually working with the malady and to protect completely other individuals in the Institute.

Two rooms on the same floor that contained our laboratory and the central media rooms were placed at our disposal for the housing of animals. The openings into the animal rooms were so fixed that no cracks between the doors and windows and their frames, large enough to permit the passage of insects, remained. Furthermore, the doors and windows were doubly screened with coarsely and finely meshed wire. Thus the mechanical spread of the virus by mice and insects was absolutely prevented. One room housed the animals known to be infected, while the other was used for animals inoculated with material undergoing tests for the presence of virus. In the "clean room" the animals were caged in individual units and workers before going from one section to another washed their gloved hands with 5 per cent lysol.

All cages were cleaned and then sterilized with 5 per cent lysol 3 times a week as well as between experiments. Droppings and bedding were collected in large covered cans containing 5 per cent lysol. After the exterior of the cans had been washed with lysol and while the containers were still wet, they were conveyed to the incinerator into which the refuse was dumped and immediately burned. Animals to be autopsied were dipped, after death, in 5 per cent lysol and wrapped in towels moistened with the same solution before being taken to the laboratory. Finally, the floors were cleansed each day with lysol and allowed to remain wet.

The cleaning of the cages and rooms and the examination of the sick animals constituted the most dangerous duties connected with the work. This is true, because the virus probably enters man through the upper respiratory tract, *i.e.*,

the conjunctival sac, nose, and mouth. When the work was begun, however, this fact was not definitely known. Consequently a costume to prevent all modes of infection was devised. A photograph of this costume is shown in Fig. 1. The uniform consists of air-tight goggles, the lenses of which were treated with a patented preparation of soap and glycerol to prevent steaming, a mask made of several thicknesses of gauze to cover the nose and mouth over which was placed a respirator frame, a hood covering the head and shoulders (the goggles were held in the hood by firmly tied draw-strings), a surgeon's gown over which was placed a heavy rubber apron, heavy rubber obstetrical gloves reaching to the elbows, and heavy rubber boots long enough to cover the legs as far up as the knees. When handling the animals, the workers wore heavy leather gloves wet with lysol in order to prevent injury to the hands and destruction of the rubber gloves. As is well known it is almost impossible to prevent the inhalation of dried infectious material unless a gas mask is used. The costume employed by us was fairly efficacious chiefly because of the dead space created by the respirator frame placed between the gauze mask and hood. When working with the virus in the laboratory, we wore goggles, a gauze mask, a surgeon's gown, and rubber gloves. The parts of the costume made of cloth, upon being removed, were dipped in lysol solution before being sent to the laundry. Rubber portions of the costume were sterilized either by boiling or by immersion in lysol solution.

All containers with liquid media were inclosed in tin cans while being incubated. Petri dishes with cultures were sealed by means of strips of rubber cut from Ford inner tubes. These measures were used to prevent the entry of insects into the plates and the consequent spread of infection, and to protect other workers using the incubator.

Three people were actively engaged in the work, while 5 other individuals were employed in different capacities in the laboratory. Furthermore, our laboratory and animal house are the most centrally located ones in the Institute. The measures we used prevented a general outbreak of psittacosis, but, for some reason not known to us, they were insufficient to protect one of the doctors studying the disease. He came down with it. We believe that the infection took place by way of the upper respiratory tract, the portal of entry most difficult to protect.

EXPERIMENTAL

In the experimental work concerning psittacosis in parrots our chief interests were centered around the portal of entry of the virus, the distribution of the incitant in the body, and the portal of exit of the active agent. Information regarding these matters seemed essential for an understanding of the spread of the disease from bird to bird, and from birds to man. Incidentally, we were anxious to learn something of the clinical and pathological manifestations of the disease in parrots.

From examination of Text-fig. 1, one learns that Krumwiede demonstrated that emulsions of mixed organs or filtrates of these emulsions administered intraorally or intramuscularly were capable of infecting birds. Furthermore, he showed that an emulsion of spleen alone injected intramuscularly was infectious. It remained for us to determine whether the virus is in the blood, in the liver, in the nasal secretions, and in the feces.* For this purpose, the following experiments were performed.

Parrot N received intramuscularly 1 cc. of a 10 per cent spleen emulsion from Parrot M, *Feb. 22, 1930*. Transferred to Rockefeller Institute, *Mar. 3*, at which time it was sick. *Mar. 5*, stools loose, feathers ruffled. *Mar. 6*, died and was autopsied immediately. Lungs negative; purulent (sterile) pericarditis; liver enlarged and friable; spleen large and soft; intestines a dusky reddish color.

Parrot N_1 received, *Mar. 8*, intramuscularly and intraorally 3 cc. and 1.5 cc. respectively of a 10 per cent liver emulsion from Parrot N. *Mar. 10*, feathers rough, stools loose. *Mar. 20*, feathers rough, stools less watery, thin frothy discharge from nose. *Mar. 26*, bird seems well again. *May 27*, reinoculated intranasally with stool from Parrot 106, and found to be immune.

Parrot K, *Feb. 15, 1930*, inoculated intraorally and intramuscularly with organ filtrate from Parrot F. *Feb. 17*, sick. *Feb. 21*, transferred to Rockefeller Institute, feathers ruffled and stools loose. Chloroformed and autopsied immediately. 2 cc. of blood drawn from the heart: half cultured and found free from ordinary bacteria, half used for inoculation of Parrot 83. Lungs and heart appear normal. Liver shows on the surface several yellowish white lesions. Spleen enlarged; intestines a dusky reddish color. Cultures of spleen, liver, and heart muscle remained sterile.

Parrot 84, *Feb. 21*, received intramuscularly 2 cc. of a 10 per cent liver, spleen, and heart emulsion from Parrot K. *Feb. 25*, looks sick, eats poorly. *Feb. 28*, weak, falls from perch. *Mar. 15*, seems much better. *April 3*, very sick again. *April 6*, very thin and weak, stools loose. Died 12 noon and was autopsied immediately. Lungs and heart normal in appearance. Liver very large; over its surface and throughout the organ numerous whitish yellow areas noted. Liver not very friable and seems to have more connective tissue than normal. Spleen very large (tenfold increase in size) and soft. Cultures of liver, spleen, and lung remained sterile.

* We knew that the virus had been demonstrated in the droppings from infected parrots (Armstrong, C., McCoy, G. W., and Branham, S. E., *Pub. Health Rep., U. S. P. H.*, 1930, **45**, 725). The fact, however, that the inciting agent is found in droppings collected from the floor of cages is not definite evidence that the virus is excreted in the feces, because the droppings might become contaminated with virus after passage from the body.

Parrot 83, Feb. 21, inoculated intramuscularly with 1 cc. of blood from Parrot K. *Feb. 26,* seems slightly ill, feathers somewhat ruffled. *Mar. 10,* bird died rather unexpectedly, inasmuch as it had never seemed very sick. Autopsy showed normal lungs, liver, and brain, purulent (sterile) pericarditis, and friable spleen. Cultures of heart muscle, liver, and spleen remained sterile.

Parrot 86, Mar. 12, received 2.5 cc. and 0.5 cc. of an emulsion of liver and spleen intramuscularly and intraorally respectively. Remained well until *Mar. 20,* when for the first time it looked sick, feathers roughened, stools loose. *Mar. 23,* better, stools formed. *Mar. 29,* very sick again, weak, feathers rough, stools loose. *Mar. 30,* chloroformed and autopsied immediately. Emaciated, lungs normal, no pericarditis, liver large and friable, spleen twice normal size and soft, intestines injected, brain normal. Cultures of lungs, liver, and spleen remained sterile.

Parrot 91, Mar. 30, inoculated intramuscularly with 4 cc. of a 10 per cent liver and spleen emulsion from Parrot 86. Also fed 1 cc. of the emulsion. *Apr. 7,* sick for the first time. *Apr. 11,* sick, stools loose. *Apr. 18,* very sick, stools watery, stools collected for filtration experiment. *Apr. 19,* died and was autopsied immediately. Not emaciated. Lungs fairly normal, small hemorrhages in visceral pericardium. Liver large and friable; throughout the organ numerous yellowish white areas of varying size (Fig. 4). Spleen 3 or 4 times normal size and spotted (Fig. 3). Intestines injected. Cultures of lungs showed several kinds of bacteria. Cultures of liver and spleen remained sterile.

Parrot 95, Apr. 18, stool collected from Parrot 91 was diluted with Locke's solution and filtered (half-hour) through a Berkefeld V. It was again filtered (10 minutes) through another Berkefeld V. The filtrate was free from ordinary bacteria. Of this filtrate Parrot 95 received 2 cc. intramuscularly, 1 cc. intraorally, and 5 cc. in its drinking water. *Apr. 26,* stools loose for first time. *May 8,* bird has gradually become worse; weak, eats poorly, feathers roughened. *May 14,* died. Immediately after death, material was collected from nose. *Autopsy:* Lungs normal; heart covered with a flaky exudate; liver large, friable, and mottled with yellowish areas of varying size; spleen 3 times normal size; flaky exudate over spleen and liver. Cultures of liver, spleen, and exudate remained sterile.

Parrot 96, Apr. 19, received intraorally and intranasally 1 cc. of a 10 per cent liver and spleen emulsion from Parrot 91. 3 cc. of the emulsion was also put on the food and in the drinking water. *Apr. 24,* stools loose. *Apr. 29,* bird very sick and weak, stools watery. *Apr. 30,* bird worse, chloroformed. After death a whitish material came from nose (probably from procrop also). The bird was held over a Petri dish into which the material was allowed to drop. *Autopsy:* Lungs normal; no pericarditis; liver fatty and friable, studded with numerous white spots; spleen 5 times normal size, pale and friable; intestines normal; brain injected. Cultures of liver and spleen remained sterile. Spleen was contaminated while being removed. Cultures of nasal secretions showed no non-lactose-fermenting bacilli.

Parrot 98, Apr. 30, 0.25 cc. of the nasal secretions collected from Parrot 96 were

dropped into the nose of Parrot 98. *May 2*, stools loose, but bird seems to be in fairly good condition. *May 5*, bird died rather unexpectedly. *Autopsy*: Hemorrhages in pericardium; lungs normal; liver large, fatty, friable, and studded with whitish spots; spleen 5 times the normal size and friable. Increase in the amount of peritoneal fluid. Cultures of this fluid remained sterile. Anaerobic and aerobic cultures of the spleen and the aerobic cultures of the liver showed no bacteria. From the anaerobic cultures of the liver a small Gram-negative influenza-like bacillus, that grew on blood agar and not on plain agar, was obtained.

Parrot 105, *May 14*, Parrot 95, immediately after death, was suspended, head down, over a Petri dish. 0.25 cc. of a thick whitish material were collected in this manner, and, having been diluted with a small amount of Locke's solution, were instilled in the nose and mouth of Parrot 105. *May 16*, stools slightly loose. *May 22*, stools watery, bird quiet, feathers roughened. *July 7*, the bird has been having recurring attacks of diarrhea. In spite of these attacks the parrot seems in good condition. Chloroformed and immediately autopsied. Not emaciated. Lungs and heart normal. Liver is extraordinary: the organ is adherent to surrounding structures, left lobe reduced to an unrecognizable small mass of scar tissue; right lobe about one-half normal size, mottled, rubbery, sectioned with difficulty. An a-p section through the whole lobe shows scar tissue in the center with normal liver tissue posteriorly. The appearance of the tissues suggests a healing or a chronic psittacosis infection. Spleen small with a whitish thickened capsule. Intestines adherent to each other in a manner suggesting a healing peritonitis. Duodenum red and inflamed. Cultures of liver and spleen remained free from ordinary bacteria.

Parrot 106, *May 14*, after Parrot 95 was dead and when its feathers had been plucked, the bird was dipped in 5 per cent lysol. Then the lysol was washed off with alcohol. By means of a sterile catheter attached to a syringe, 5 cc. of Locke's solution were injected into the cloaca and then withdrawn again. In this manner, fecal material was collected free from outside contaminants. 1 cc. of this material was instilled in the nose of Parrot 106. *May 19*, stools loose. *May 22*, bird worse, feathers roughened. *May 25*, bird very weak, right eye closed, stools watery. *May 26*, found dead. *Autopsy*: Bird emaciated. Pericardium, heart, and lungs normal. Liver large, fatty, friable; along interlobar fissure are yellowish areas of necrosis surrounded by bright red hemorrhagic zones, numerous similar but smaller areas ranging from pin points to peas in size were scattered through the organ. Spleen 4 times normal size and friable. Cultures of liver revealed no ordinary bacteria.

Parrot 107, *May 28*, stool collected from Parrot 106 in a manner similar to that employed with Parrot 95 (see Parrot 106 for details). 1 cc. of this fecal material was instilled in the nose and mouth of Parrot 107. *May 30*, stools loose. *June 2*, bird worse. *June 11*, the bird has been gradually getting worse; weak, feathers roughened, severe diarrhea. Chloroformed and autopsied immediately. Lungs normal. Pericardium contains 0.5 cc. of purulent-looking, sticky exudate. Liver

smaller than normal and shows numerous necrotic zones. Spleen 3 times normal size and friable. Cultures of liver remained sterile. Smears from liver and spleen showed no "minute bodies." Preparations from the pericardial exudate stained according to a modification of Castaneda's methylene blue safranin method* revealed numerous "minute bodies," of the type first described by Levinthal (6).

The experiments detailed above are summarized in Text-fig. 1. From an examination of the results, certain facts become obvious. In the first place, the virus of psittacosis is found in the stools, in a mixture of nasal secretions and material from the procrop, in the blood, in the liver, and in the spleen of infected birds. Moreover, parrots are capable of being infected by intramuscular, intranasal, or intraoral inoculations of the virus. These facts indicate the manner in which the disease spreads from bird to bird, and also suggest the source of infection for man.

The clinical picture of psittacosis in birds varies. The disease may be acute, running its course in a few days, or it may be chronic, enduring for several months. The birds may die suddenly without showing appreciable signs of illness. As a rule, however, loss of weight, roughening of the feathers, weakness, watery stools, and discharge from the nose are observed during the course of the disease. In our experience, the majority of the infected birds died. The gross pathology is characterized by an occasional sterile pericarditis, enlarged, fatty livers, many of which show areas of necrosis (Fig. 4) or infarction, enlarged friable spleens (Fig. 3), and injected intestines. Regardless of the mode of inoculation, none of the parrots revealed changes in the lungs that might be attributed to psittacosis.

Microscopic Pathology

Spleen.—Changes in the lymphoid follicles vary from slight alterations to an almost complete obliteration of their normal architecture (Figs. 5 and 6), while

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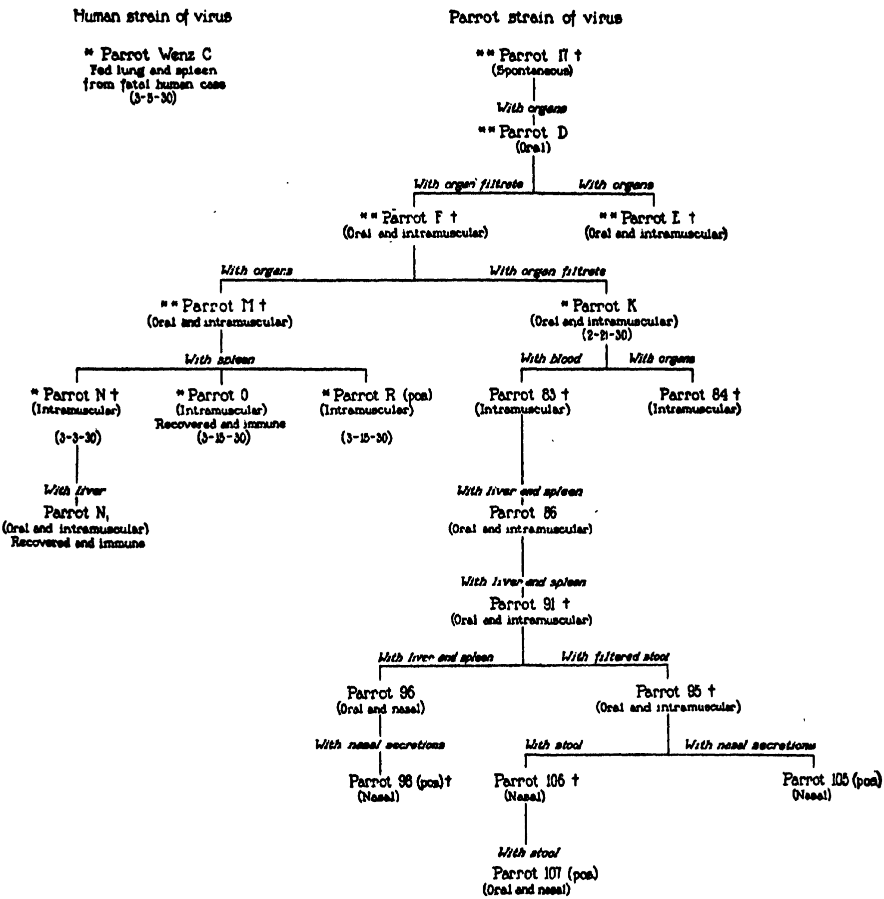
	cc.
* Phosphate buffer pH 7.0	95
Formalin	5
Loeffler's methylene blue	10

Stain 2 minutes, rinse with tap water, and quickly counterstain with aqueous safranin.



TEXT-FIG. 1. Diagrammatic representation of the experimental work concerning psittacosis conducted in parrots. The doubly starred birds were handled only by Krumwiede, the singly starred parrots were handled both by Krumwiede and by us, the birds without stars were handled only by us. † indicates that the bird died.

the reticular and sinus structures are well preserved. The organ is infiltrated with wandering phagocytic cells with vacuolated cytoplasm containing amorphous débris, pigment, and globules of fat. The increase in the size of the spleen seems to be due to the content of blood and the enormous number of mononuclear cells.

*Liver.**—The characteristic lesion of psittacosis in parrots consists of multiple discrete areas of necrotic liver cells irregularly distributed throughout the organ, but with a tendency to be more numerous near its periphery. The condition appears to have its onset in the death of isolated liver cells or groups of cells; the cytoplasm becomes acidophilic and granular, and shrinks from contact with other cells. The nuclei become hyperchromatic and pyknotic, and eventually disappear completely. At this stage, mononuclear phagocytes and a few polymorphonuclear cells surround and infiltrate the lesion. As the process progresses, the necrotic liver cells disintegrate, leaving strands of acidophilic, hyaline material which may or may not show collections of leucocytes and depositions of fibrin (Fig. 7). When necrotic areas penetrate to the surface of the liver, accumulations of inflammatory cells are seen under Glisson's capsule, and an extension of the process leads to the perihepatitis and peritonitis that are encountered. Around the zones of necrosis, proliferation of liver cells, indicated by mitotic figures, occurs. Within and around the lesions, cells, for the most part "endothelial leucocytes," filled with the "minute psittacosis bodies," are also found (Fig. 2).

Throughout the liver there is a proliferation of Kupffer cells and focal accumulations of wandering mononuclear cells of the same general type. Some of these have a highly vacuolated cytoplasm containing masses of fat. Many plasma cells are also present.

The bile ducts do not escape injury. Within the necrotic areas, they are dilated, and at times contain numerous mononuclear phagocytes. The cells that form the walls of the ducts may undergo necrosis, becoming granular with pyknotic or missing nuclei. In chronic and in healing lesions, irregularly shaped collections of hepatic cells separated from each other by various sized bile ducts undergoing proliferation and surrounded by lymphocytes and fibrous tissue (Fig. 8) are observed. The dilatation of the bile ducts and the character of the lesions in the later stages of the disease which resemble those produced by ligation of the common duct, lead one to believe that occlusions of the biliary system in the parrot may occur in some manner as the result of an infection with the virus of psittacosis.

Striking alterations in the vascular channels are not usually seen, but in certain

* The anatomy of the parrot's liver differs from that of mammalian livers in that the bile duct draining the left lobe enters directly into the duodenum. There are also differences in the microscopic appearances of the two kinds of livers. For example, lobulation in the parrot's liver is not well developed and arrangement of the parenchymal cells in chains occurs only to a slight extent. Moreover, the bile ducts, which in mammals are invariably associated with blood vessels, may at times be found unassociated with such structures in the parrot.

TABLE I

Summary of Reinoculation Experiments in Parrots

Parrot No.	1st inoculation Material Route, date	Virus in inoculum*	Course after inoculation	Time between inoculations	Reinoculation Material Route, date	Course after inoculation	Autopsy	Active immunity + or −
				days				
O	Parrot spleen i.m. 2-22-30	+	Sick 1 mo.	95	All inoculated in manner identical with control Parrot 107 5-28-30	Negative	Lived	+
McG	Human blood i.m. 3-7-30	−	Negative	82		Died on 9th day	Psittacosis	−
N_1	Parrot liver Oral and i.m. 3-8-30	+	Sick 3 wks.	81		Occasional mild diarrhea	Lived	+
89	Mouse organs Oral and i.m. 3-28-30	+	Sick 1 mo.	61		Negative	Lived	+
92	Human blood Oral, i.p., i.m. 4-3-30	−	Negative	55		Typical illness, killed after 9 days	Psittacosis	−
93	Human nasal washings Oral 4-3-30	?	Very slight diarrhea	55		Typical illness, killed after 6 days	Psittacosis	−
107	Control—Inoculated by oral and nasal instillation of 1 cc. of stool from Parrot 106, sick with psittacosis. Was sick 48 hrs. after inoculation; ran fulminating course; was killed when moribund on 14th day. Autopsy showed typical psittacosis							
102	Human lung Oral and i.m. 5-9-30	−	Negative	31	Mouse organs Oral and i.m. 6-9-30	Typical illness, killed on 8th day	Psittacosis	−

Control—Material used for reinoculation of Parrot 102 produced psittacosis in Mice WC_{24} and Monkeys H, I, and J.

* Presence or absence of virus in inoculum determined in addition by other animal inoculations.

cases fibrin thrombi, involving portal vessels, are found. In most instances, only small vessels are occluded, but occasionally a large branch is involved. The question as to whether the areas of necrosis result from vascular thromboses or whether the thromboses are caused by necrosis cannot be definitely answered. But the evidence is such that it seems unlikely that much of the necrosis is the result of vascular occlusions.

Immunity

Having investigated the portal of entry and exit of the virus in parrots and upon concluding the study of the clinical and pathological pictures of the disease in its natural host, we then became interested in determining whether the birds that had recovered from psittacosis were resistant to reinfection.

For the work on active immunity, 7 parrots were available; 3 had recovered from psittacosis experimentally induced 61–91 days prior to the reinoculation, 3 had been inoculated 31–82 days previously with material subsequently shown to be free of virus, 1 had received intraorally either a very small amount of virus or none at all. In any event, the last bird mentioned evidenced few if any signs of illness following the first inoculation. 6 of the birds (O, McG, N, 89, 92, 93) and a control received intranasally and intraorally 1 cc. each of an unfiltered stool from Parrot 106 sick with psittacosis. The seventh parrot in the group (102) was tested for immunity by means of a virus-containing emulsion from mice. The 3 parrots (O, N, 89) that had recovered from a previous infection lived, while the others developed psittacosis and reacted in a manner similar to that of the control. The results of these experiments are summarized in Table I.

From the experiments described above and summarized in Table I, it is obvious that parrots are actively immune following an attack of psittacosis.

DISCUSSION

The results of our investigations concerning psittacosis experimentally induced in parrots need few comments. It seems advisable, however, to emphasize again the danger of studying the disease in parrots. Due to the presence of virus in the nasal secretions and feces of infected birds and because of the parrot's filthy habits, to protect oneself against the entry of dried virus into the upper respiratory tract is extremely difficult. In view of this fact, and since mice are suitable for diagnostic and experimental work, as demonstrated in the second paper of this series, investigations with parrots should be limited as much as possible.

The "minute bodies" found in exudates and in certain infected organs were first described by Levinthal (6) who thinks that they are of etiological significance and probably represent small bacteria of a nature similar to that of *B. tularense.* Lillie (7) believes these structures are *Rickettsiae* and proposes for them the name *Rickettsia psittaci.* Coles (8) speaks of them as "x-bodies" and is also of the opinion that they probably constitute the causal agent. We have experienced no difficulty in finding these bodies in some animals, while in others extensive search has failed to reveal them. When present they take stains with ease, are Gram-negative, and closely resemble minute microorganisms with a diameter of about 0.2μ. As yet, however, no one has succeeded in cultivating them on ordinary laboratory media, and their exact nature and relation to psittacosis is still an open question.

CONCLUSIONS

1. The virus of psittacosis is present in the nasal secretions, feces, blood, spleen, and liver of an infected parrot.
2. Parrots are susceptible to intraoral, intranasal, or intramuscular inoculations of the virus.
3. The most constant pathological changes produced by psittacosis in parrots occur in the spleen and liver. The lesions exhibited in the latter organ consist of areas of necrotic liver cells and damage to bile ducts. In no instance, in our experience, were lesions observed in a parrot's lungs comparable to those found in the lungs of men.
4. "Minute bodies" similar to those described by Levinthal and others were found in many, but not in all of the infected birds.
5. Parrots that have recovered from one attack of psittacosis exhibit an active immunity against reinfection.

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EXPLANATION OF PLATES

Plate 5

Fig. 1. Photograph of costume worn in animal rooms. A parrot with psittacosis is perched on the left wrist of the worker. The other bird is normal.

Fig. 2. "Minute bodies" in mononuclear cells of the liver. ×1700.

Fig. 3. An enlarged and mottled spleen from a parrot with psittacosis. ×1.

Fig. 4. Liver from a parrot infected with psittacosis. The necrotic areas are white. ×1.

Plate 6

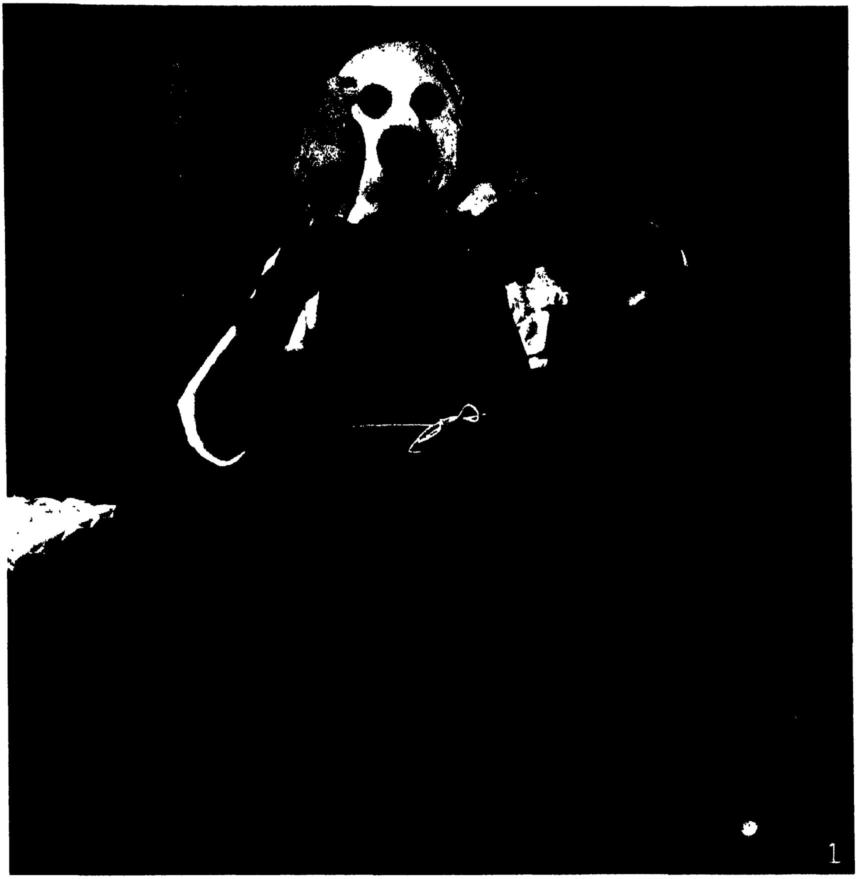
Fig. 5. Section from a parrot's spleen injured by the virus of psittacosis. Normal architecture destroyed. ×170. Eosin and methylene blue.

Fig. 6. Normal parrot's spleen. Compare with Fig. 5. ×170. Eosin and methylene blue.

Plate 7

Fig. 7. Section from a liver of a parrot with psittacosis. Early lesions showing degeneration of liver cells and depositions of fibrin. ×115. Eosin and methylene blue.

Fig. 8. Section from a liver late in the disease, showing proliferation of bile ducts, infiltration of mononuclear cells, and deposition of connective tissue. ×450. Eosin and methylene blue.



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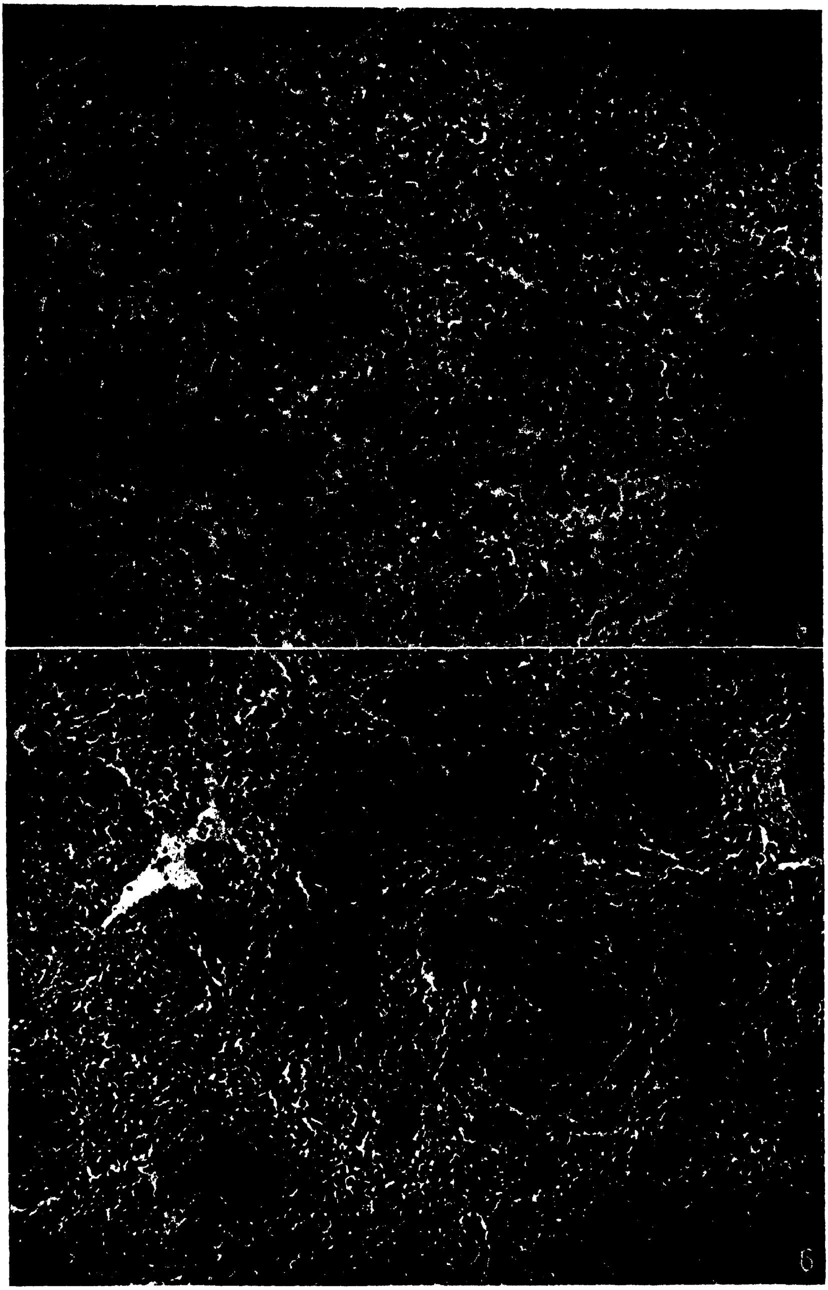
Photographed by Louis Schmidt

(Rivers *et al.*: Psittacosis. I)



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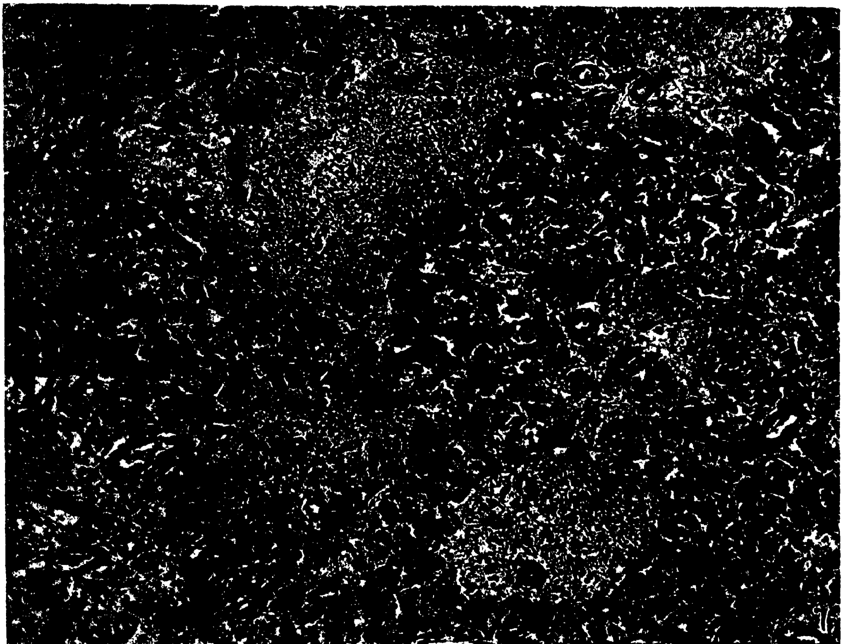

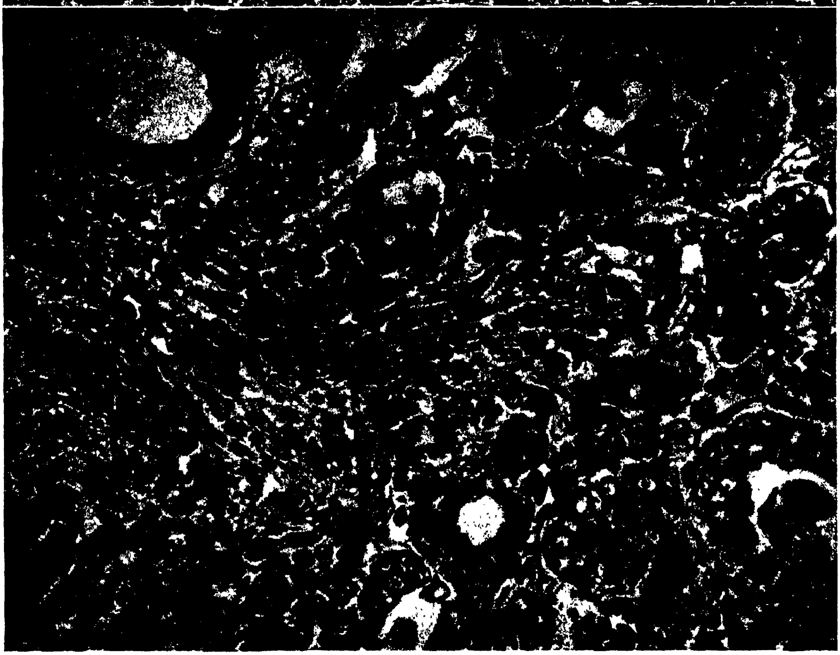

Photographed by Louis Schmidt

(Rivers *et al.*: Psittacosis. I)

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PSITTACOSIS

II. EXPERIMENTALLY INDUCED INFECTIONS IN MICE

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PLATES 8 TO 10

(Received for publication, April 1, 1931)

Krumwiede and his coworkers (1) were the first to report that mice are capable of being experimentally infected by intraperitoneal inoculations of the virus of psittacosis derived from a parrot. Furthermore, they showed that the active agent can be transmitted from mouse to mouse in series. These observations were important because they provided a readily available experimental animal and one much less dangerous to handle than are parrots. Consequently, when Krumwiede and his associates discontinued their work and gave us two strains of virus, observations on psittacosis infections in mice were immediately begun. The object of this communication is to describe some of our experiences encountered during the course of these investigations.

Methods and Materials

Virus.—Active agents from 3 sources were used. One strain (Parrot N) came from a bird (see first paper in this series) infected with virus originally obtained from a sick parrot. The second strain (Wenz C or WC) was secured from a parrot inoculated with material from a patient who had died of psittacosis. The third virus was obtained from the sputum of a patient (G. P. B.) during the course of a psittacosis infection contracted in the laboratory (2). From 2 sources in the mice, *viz.*, brain, pooled liver and spleen, the virus was secured for passages and experimental purposes. The organs used were always tested for the presence of ordinary aerobic and anaerobic bacteria.

Inoculation.—The mice were usually inoculated intraperitoneally or intracerebrally with 0.5 cc. and 0.025 cc. respectively of organ emulsions. The technique of intracerebral injections will be described in detail later in the paper.

Clinical Signs of Infection in Mice.—In animals inoculated intraperitoneally, the time of onset of the illness varied with the amount and potency of the virus.

Loss of appetite and weight, and roughening of the fur were constant signs of infection. A few animals died during convulsive seizures. The elapse of time between the inoculations and the death of the animals varied, *e.g.*, many mice died within 48 hours, others succumbed after 3 weeks, and a few recovered. The clinical picture of the disease in mice intracerebrally inoculated will be discussed in another part of the paper.

EXPERIMENTAL

Three strains of psittacosis virus were studied experimentally in mice. A brief review of the findings with 2 of them will be made, after which follows a more detailed description of the work with the third. Then, the pathological changes caused by the virus in mice will be discussed. Finally, certain experiments dealing with the phenomena of immunity will be described.

Mice Infected with a Parrot Strain of Virus

The following experiments were performed to ascertain whether a parrot strain of psittacosis virus is capable of propagation in mice.

A 10 per cent emulsion of liver and spleen from Parrot N was divided into 2 portions, one of which was heated at 100°C. for 1 minute. Then 4 mice were inoculated intraperitoneally (0.5 cc. each) with the heated, and 4 more with the unheated material. None of the animals that received the heated emulsion became ill, while all of those that were inoculated with the unheated material sickened and died. An emulsion was made from the liver and spleen of one of the mice that died. Again the emulsion was divided and treated in a manner similar to that already described. The 4 mice inoculated with the heated material remained healthy, and later, when 2 of them had been sacrificed, 4 other mice were injected with an emulsion of their livers and spleens. These animals showed no illness. The mice, however, that received the unheated emulsion died in 4–7 days. By means of intraperitoneal inoculations of liver and spleen emulsions, serial passages of the virus through 7 successive groups of mice were made, and the typical clinical and pathological pictures of psittacosis were observed in the infected animals. The series was discontinued after the 7th passage.

The above group of experiments shows that a parrot strain of psittacosis virus is pathogenic for mice, and that the virus can be propagated serially in mice by means of intraperitoneal inoculations of infected hepatic and splenic emulsions. Moreover, virus-containing emulsions that have been heated at 100°C. for 1 minute and emulsions of livers and spleens from normal mice are innocuous.

Mice Infected with B-Sputum Strain of Virus

An infection incurred in the laboratory provided an opportunity of determining (1) whether psittacosis virus occurs in a patient's sputum, and (2) whether mice are suitable for diagnostic purposes.

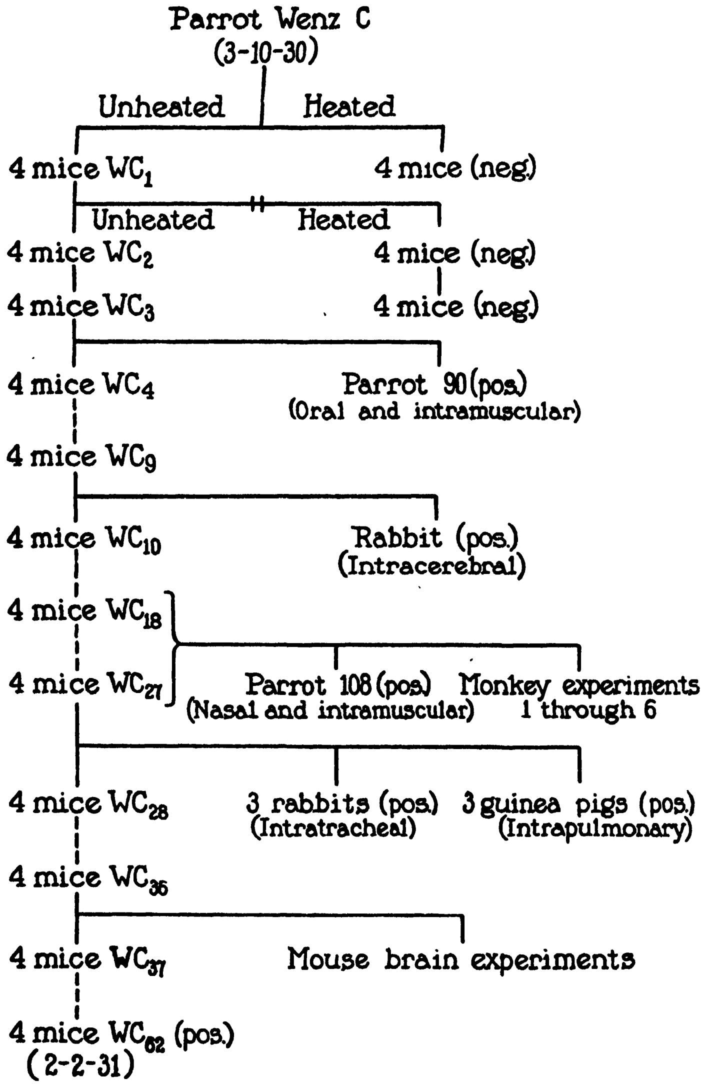
G. P. B. expectorated a mass of thick tenacious sputum on the 10th day of his illness (2). This sputum was washed and emulsified in Locke's solution. Cultures of the emulsion showed a few colonies of organisms usually found in the throat. Apr. 10, each of 4 mice received intraperitoneally 0.5 cc. of the emulsified sputum. On Apr. 12th, one mouse was found dead. Since it had evidently died of a bacterial infection, no passage from it was made. On Apr. 25th, however, another mouse died, and shortly after death an emulsion of its liver and spleen, which was free from ordinary bacteria, was injected into normal mice. These animals sickened and died in 3 or 4 days. With liver and spleen emulsions passages were made in series through 13 sets of mice at which time they were discontinued. The majority of the animals died 3–7 days after inoculation and presented a typical picture of psittacosis. Some of the material used for the 4th transfer induced psittacosis when injected into Parrot 102.

The above experiments clearly indicate that mice may be used for the diagnosis of psittacosis. Furthermore, they demonstrated for the first time—and the results were confirmed by inoculation of a parrot—that the virus is present in the sputum of man infected with psittacosis. Bedson and Western (3) state that Krumwiede (1) proved the presence of virus in a patient's sputum. But the report of Krumwiede and his coworkers shows that they demonstrated the active agent in a mixture of sputum and blood. Since it is known that the virus is frequently in the blood, such an experiment does not suffice to prove its presence in the sputum. Nevertheless, it is the virus in the sputum which probably constitutes the chief source of danger in handling patients with psittacosis.*

Mice Infected with Wenz C Strain of Virus

Parrot Wenz C was inoculated by Krumwiede with an emulsion of lung and spleen from a woman who had succumbed to psittacosis.

* A small epidemic of psittacosis recently occurred in Brooklyn. Mice alone were used to obtain the virus from sputum, and autopsy material. They were adequate for diagnostic purposes. The details of this work will be published later.



TEXT-FIG. 1. Schematic portrayal of the experimental work in mice dealing with the WC strain of psittacosis virus.

Shortly after inoculation the bird was transferred to The Rockefeller Institute, where it soon became sick. From a sterile emulsion of the liver and spleen of this parrot a series of passages and experiments

in mice, schematically portrayed in Text-figs. 1 and 2, were initiated March 10, 1930.

Serial Passages by Intraperitoneal Injections of Emulsified Livers and Spleens.—In Text-fig. 1 the course of the work is graphically depicted: the mode of transfer, when not designated, consisted of an intraperitoneal injection of an emulsion of pooled liver and spleen. From the diagrammatic representation of the experiments, one ascertains that serial passage of the virus has been made on 62 occasions extending over a period of nearly a year. Moreover, it is obvious that heated emulsions of livers and spleens from infected parrots and mice and unheated emulsions of livers and spleens of normal mice did not induce psittacosis. Furthermore, it was demonstrated that the virus propagated in mice is pathogenic for parrots, rabbits, guinea pigs, and monkeys.

Passage of the virus through mice in some manner caused it to become more potent for this host.

Early in the work, 0.5 cc. of a 10 per cent organ emulsion required 4 or 5 days to kill the animals, and a few mice survived. After the 40th passage, however, such a dose was regularly fatal within 48 hours. Titrations of the virus after 60 passages gave the following results: 4 mice were inoculated with 0.5 cc. of each dilution—10 per cent, all dead in 48 hours; 1 per cent, all dead in 3–5 days; 0.1 per cent, all dead in 6 days; 0.01 per cent, all dead in 8–9 days; 0.001 per cent, all dead in 8–9 days; 0.0001 per cent, all dead in 10–11 days. Thus, it appears that the virus is now quite virulent for mice, and there is no reason to suppose that it will not retain its potency.

On the basis of the work just presented, one is justified in concluding that at least certain strains of psittacosis virus are capable of being propagated indefinitely from mouse to mouse by intraperitoneal injections of emulsified livers and spleens. Our own work with rabbits and guinea pigs, to be reported in the third communication of this series, and the experiences of different investigators in producing herpetic infections (4) and yellow fever (5) in mice by intracranial inoculations of infectious material induced us to attempt serial passages of psittacosis virus in the brains of these animals.

Intracerebral Inoculations

Technique.—Intracerebral inoculations* in mice are accomplished with ease. A 0.25 cc. tuberculin syringe fitted with a 27 gauge needle, was used. With the body of the mouse under the palm of the left hand, one grasps the head between the thumb and index finger and presses it firmly against the table. The fur is then parted over the middle of the left parietal bone by means of a small cotton swab moistened with 95 per cent alcohol. The right hand holding the syringe slowly pushes the needle through the skull, 2 mm. to the left of the midline, to a depth of about 2 mm. and injects 0.025 cc. of the inoculum. If serum, Locke's solution, or normal brain emulsion is injected in this manner, the mice appear normal within 30–60 minutes. If irritating fluids are introduced, 24 hours may be required for the return to normal.

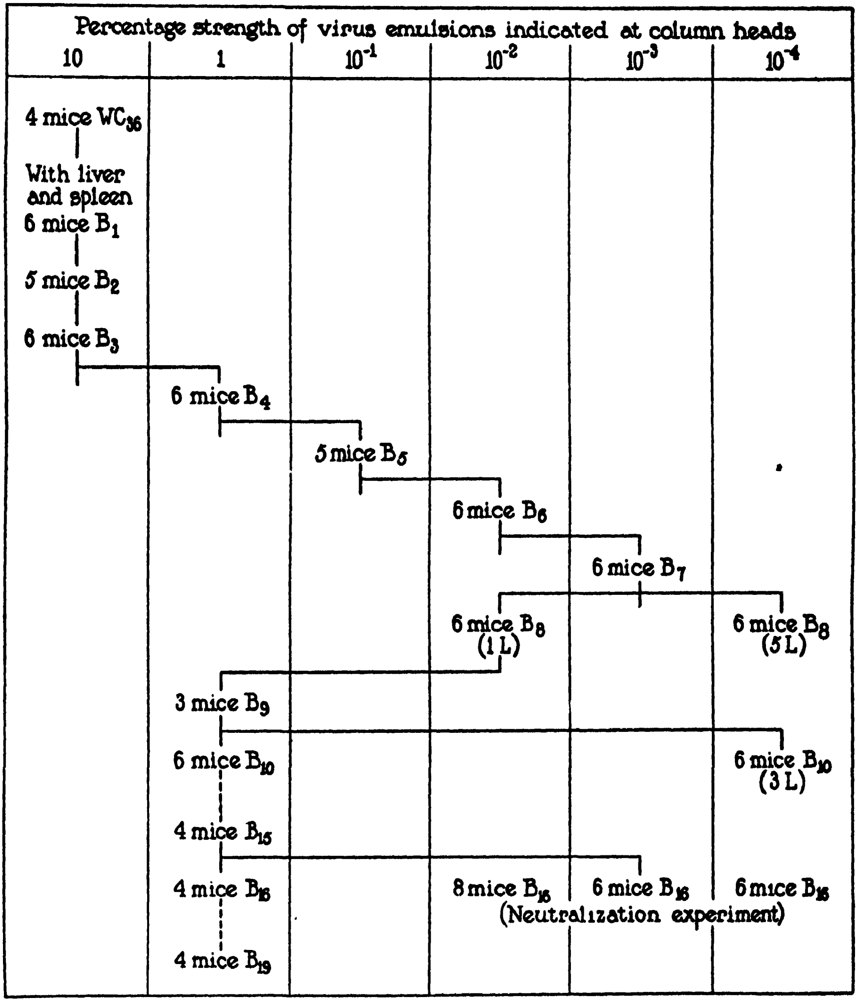
Clinical Picture and Time of Death.—Except for the mice that received the most concentrated emulsions, the animals appeared normal the day following inoculation and so remained from one to several days according to the dose. Death usually occurred within 48 hours after the appearance of signs of illness. Failure to eat and hyperirritability accompanied by roughening of the fur were the first evidences of sickness. The irritability manifested itself in exaggerated responses to different stimuli. Between the periods of motor hyperactivity, the animals sat quietly with arched backs and with heads held perpendicular to the floor. At this stage of the disease, the eyes were surrounded by red circles. Twitchings involving the jaw, eyes, and legs developed. Ataxia was pronounced and some of the mice went round in circles, or would rise up and fall over backwards. Then, generalized convulsive seizures developed and death followed. The posture in death was characteristic and striking; the head was retracted, the back was arched, the forelegs were flexed, the hindlegs and tail were extended. Immediately after death, rigor mortis developed. The time of death varied with the dilution of the inoculum. The following results illustrate this fact: 4 mice were inoculated with 0.025 cc. of each dilution—1 per cent, all dead in 40–60 hours; 0.1 per cent, all dead 3–4 days; 0.01 per cent, all dead 3–4 days; 0.001 per cent, all dead 4–5 days; 0.0001 per cent, all dead 5–7 days; 0.00001 per cent, all dead 6–7 days. The potency of emulsions varies from time to time, and some of the mice that received the higher dilutions occasionally survived.

Results

The passages of virus from brain to brain were initiated by the intracerebral inoculation of 6 mice with 0.025 cc. each of a 10 per cent emulsion of livers and spleens from mice WC_{36} (see Text-figs. 1 and 2). The results of the work portrayed in Text-fig. 2 demonstrate that the virus by intracranial inoculations of emulsified brain tissue was serially

* All operations were performed under light ether anesthesia.

propagated through 19 sets of mice. Moreover, since 0.025 cc. of a 0.0001 per cent or of a 0.00001 per cent emulsion killed a large per-



TEXT-FIG. 2. Graphic representation of experiments dealing with the serial intracranial passage of psittacosis virus in mice.

centage of the mice, it appears that the potency of the virus in the brain was equal to, if not greater than that in the livers and spleens.

Pathology

The pathological findings vary somewhat according to the mode of inoculation and the lapse of time between inoculation and death. The gross changes in the different sets of animals will be described first, after which the microscopic pathology will be discussed.

Gross Pathology of Mice Inoculated Intraperitoneally.—The mice that received intraperitoneal injections of virus-containing emulsions were emaciated and frequently had distended abdomens. On opening the abdominal cavity, one usually found an excess of sticky fluid containing flakes of fibrin. The livers were enlarged and friable; often they had undergone such extensive fatty degeneration that they had become chamois-colored; and scattered over the surface yellowish white spots, surrounded occasionally by hemorrhagic zones, were at times seen. Such spots, most frequently observed at or near the edges of the lobes, resembled areas of necrosis or minute infarcts (Fig. 5). If the mice died promptly, enlargement and fatty degeneration were the only gross evidences of involvement of the liver. The longer the animals survived after infection, the more likely were the necrotic areas in the liver to be found. The spleens were usually enlarged, very red, and friable. At times whitish spots could be seen shining through the capsule. The pancreas, adrenals, and intestines were not particularly abnormal. The kidneys showed a mild parenchymatous degeneration. When the thorax was opened, the pleura and heart were usually normal in appearance. The lungs were redder than normal, but pneumonia was seldom if ever noted. Aerobic and anaerobic cultures of the livers and spleens with a few exceptions remained sterile. When bacteria were present, they were found to be of various sorts and of no etiological significance.

Gross Pathology of Mice Inoculated Intracerebrally.—The chief difference between the mice inoculated intracerebrally and those injected intraperitoneally was the involvement of the brain. In opening the cranial cavity, one often saw a small hemorrhage beneath the scalp and a hemorrhagic spot in the left hemisphere of the brain where the injections were made. The sutures of the skull were wide and bulging, and when pieces of the bones were removed, the congested edematous brain herniated through the opening. Covering the surface of the brain and lying in spaces between the lobes was a sticky exudate. Cultures of this exudate and brain tissue remained sterile. The organs in the thorax were normal. The abdomen was not distended, but, in a certain number of the animals, gross changes were noted in the livers and spleens. The former were usually fatty, and about 20 per cent of them showed areas of necrosis. The latter were enlarged, and necrotic zones were seen in approximately 10 per cent of them.

Microscopic Pathology. Liver.—When the mice were inoculated intraperitoneally, an exudate, consisting of fibrin, polymorphonuclear and mononuclear cells, at times was seen over the surface of the liver. The pathological pictures

presented by stained sections of the liver varied with the duration of the disease. During the acute stage, a general infiltration with fine globules of fat and a granular degeneration of the liver cells were present (Fig. 12). Isolated groups of cells with no apparent relation to the structural landmarks of the organ, underwent an acidophilic degeneration and finally disappeared leaving a pink reticulated mass which rapidly became infiltrated with polymorphonuclear cells (Figs. 6–9). If the animals survived more than several days or if they were killed during the period of convalescence, the liver cells were almost normal and the polymorphonuclear infiltrations had been replaced by compact nests of mononuclear cells (Figs. 10, 11). The cause of the necrosis is not known. In addition to the necrotic lesions, the sinuses of the liver contained large numbers of mononuclear cells, many of which were apparently derived from Kupffer cells.

Animals inoculated intracerebrally rarely exhibited a peritoneal exudate, but in a general way showed a type of pathology of the liver similar to that seen in the intraperitoneally infected mice with the exception that necrosis was not so prominent a part of the picture—occurring in about 20 per cent of the cases.

The areas of necrosis in many respects resemble those found in human beings with typhoid fever and in mice infected with mouse typhoid. There are certain definite differences, however. For example, in the livers infected with the virus of psittacosis vascular thromboses are not an obvious part of the picture, while in mouse typhoid they are prominent. Moreover, the livers from mice with psittacosis are free from ordinary bacteria while this is not true of livers infected with mouse typhoid.

Spleen.—Stained sections from spleens (Fig. 1) of normal mice showed large well defined lymphoid follicles sharply separated by splenic pulp. Preparations from spleens damaged by psittacosis, however, did not stain well. The lymphoid follicles were less definite in outline and contained many necrotic or hyaline cells both centrally and peripherally situated (Figs. 2, 3). In the pulp, particularly around the periphery of the organ, individual necrotic cells or groups of them, and wandering phagocytic cells filled with amorphous débris were present. In the follicles and also in the pulp, the areas containing the degenerated or necrotic cells were infiltrated with polymorphonuclear elements and resembled minute abscesses (Fig. 4). Cultures from these spleens, however, remained free from ordinary bacteria. About 10 per cent of the spleens from mice intracerebrally infected also revealed areas of necrosis similar to those just described.

Brain.—Brains from intraperitoneally infected mice appeared normal, while those from animals intracerebrally inoculated showed a meningo-encephalitis. The reaction, chiefly meningeal, was characterized by an extensive exudate composed of polymorphonuclear and mononuclear cells. The blood vessels extending into the brain were surrounded by cellular infiltrations.

"Minute Bodies."—The minute coccobacillary structures originally described by Levinthal (6) and later designated as *Rickettsia psittaci* by Lillie (7), and as

"x-bodies" by Coles (8) were found in sections* and smears† of exudates and organs from mice infected with psittacosis. They were abundant in the peritoneal and meningeal exudates and in the livers and spleens. Not always, however, were they found in material known to be infectious. Furthermore, livers and spleens from mice that died within 48 hours after infection showed either none or only a few of the bodies, while organs from animals that survived a number of days contained many such structures. These bodies were small cocci or short bacillary structures, 0.2–0.25μ in diameter or in length respectively. They occurred singly or in clusters intracellularly or extracellularly. For the most part, they were seen in the cytoplasm of mononuclear cells which were greatly distended by them. The nature of the bodies is not definitely known, but they appear to be minute organisms, and the idea, held by certain workers, that they represent the etiological agent of psittacosis deserves serious consideration.

Immunity

It seemed advisable to determine whether mice that had recovered from psittacosis were actively immune. Furthermore, an effort was made to ascertain whether the mouse can be used for testing the presence of protective antibodies in the sera of human beings convalescing from psittacosis. In this way, it was hoped that an easy experimental method might be found for the diagnosis of certain obscure conditions in man, thought to be psittacosis.

Active Immunity.—53 mice that had survived their original inoculation for varying periods of time were reinfected, 42 intraperitoneally and 11 intracerebrally. Of the 53 animals, only 5 lived for as long as 2 weeks. The others sickened and died in a manner identical with that of the controls. The reinfecting dose was 0.5 cc. of a 10 per cent organ emulsion that was employed for regular passages.

Protection Experiments.—4 groups of experiments were performed, involving 16

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* In sections the bodies are colored blue with eosin and methylene blue and with Giemsa's stain.

† For the study of smears, a modification of the stain used by Castaneda in Zinsser's laboratory for the demonstration of Mooser bodies (*Rickettsiae*) in Mexican typhus was used. This stain colors the minute bodies blue and the cells and albuminous material pink.

	cc.
Phosphate buffer pH 7.0	95
Formalin	5
Loeffler's methylene blue	10

Stain 2 minutes, rinse with water, counterstain for a few seconds with aqueous safranin.

separate tests and 7 controls. Each test was carried out in 4 mice. Consequently 92 mice were used. Convalescent sera,* collected from 10 different patients 6–8 weeks after subsidence of fever, were tested. Serum from a rabbit that had recovered from an intracerebral inoculation of virus was also used. Normal human serum, normal rabbit serum, and Locke's solution were employed as controls. Each mouse was given intraperitoneally 0.5 cc. of normal serum, convalescent serum, or Locke's solution from 4–24 hours before the intraperitoneal administration of 0.5 cc. of a virus-containing emulsion. The animals in the test group showed no evidence of having been protected, inasmuch as they died as promptly as did the controls.

Neutralization Experiments.—One neutralization experiment, involving 18 mice, divided into 3 groups of 6 each, was performed. The serum tested was collected from a patient (G. P. B.) 10 days after subsidence of fever. The controls were Locke's solution, and normal human serum pooled from 5 individuals. The test and control sera, and Locke's solution respectively were mixed with an equal volume of virus-containing brain emulsion diluted to a percentage strength of 10^{-2}, 10^{-3}, 10^{-4}. The mixtures were incubated at 37°C. for 1 hour and then chilled for 1 hour, after which 0.025 cc. of each were intracerebrally injected in mice. All the mice responded to the inoculations in a similar manner. Thus, from this experiment no evidence that convalescent human serum contains an appreciable amount of neutralizing substances for psittacosis virus was obtained.

From the results of our investigations in mice concerning immunity to psittacosis it appears that little if any active resistance develops in these animals following one attack of the disease, that convalescent human serum does not protect them against virus administered 4–24 hours later, and, finally, that convalescent human serum possesses relatively small amounts of neutralizing antibodies. Although our experiments seem to indicate that no active immunity develops in mice, the evidence is not conclusive, inasmuch as an overwhelming dose of virus was used for reinoculation. Further work, therefore, is being conducted to obtain more accurate information.

SUMMARY AND CONCLUSIONS

The work presented in this communication concerning psittacosis in mice confirms Krumwiede's observations that mice inoculated intraperitoneally with emulsified livers and spleens containing the virus develop the disease and that the malady can in this way be passed serially through a number of mice. Furthermore, it has been

* Supplied by Drs. Amoss, Krumwiede, and Wadsworth.

shown that mice are susceptible to the virus administered intracerebrally and that the active agent can be propagated indefinitely by means of brain to brain inoculations. Moreover, by the use of mice, the presence of the virus of psittacosis in the sputum of a patient with the disease has for the first time been demonstrated. It follows that the mouse is available for diagnostic purposes.

The pathological findings in infected mice consist of enlarged fatty livers that frequently show areas of necrosis infiltrated with polymorphonuclear and mononuclear cells; enlarged spleens with areas of necrosis and cellular infiltrations involving the pulp and lymphoid follicles; and, finally, in intracerebrally infected animals, a meningo-encephalitis. The "minute bodies" described by other observers were not found in all animals, but they were seen with sufficient frequency in smears of peritoneal and meningeal exudates and in smears and sections of livers and spleens to demand serious consideration as the possible etiological agent of the disease.

Neutralizing and protective antibodies were not found in convalescent human sera when the mouse was used as the test animal.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Section from spleen of a normal mouse. Compare with Fig. 2. ×85. Eosin and methylene blue.

FIG. 2. Section from spleen of mouse with psittacosis. Lymphoid follicles and pulp extensively involved. Compare with Fig. 1. ×85. Eosin and methylene blue.

FIG. 3. Small area of hyaline necrosis in lymphoid follicle of spleen. ×375. Eosin and methylene blue.

FIG. 4. Lesion in splenic pulp; area of hyaline necrosis infiltrated with polymorphonuclear leucocytes. ×375. Eosin and methylene blue.

PLATE 9

FIG. 5. Liver from mouse with psitacosis. The organ has undergone fatty degeneration, and is mottled with areas of focal necrosis. ×1.

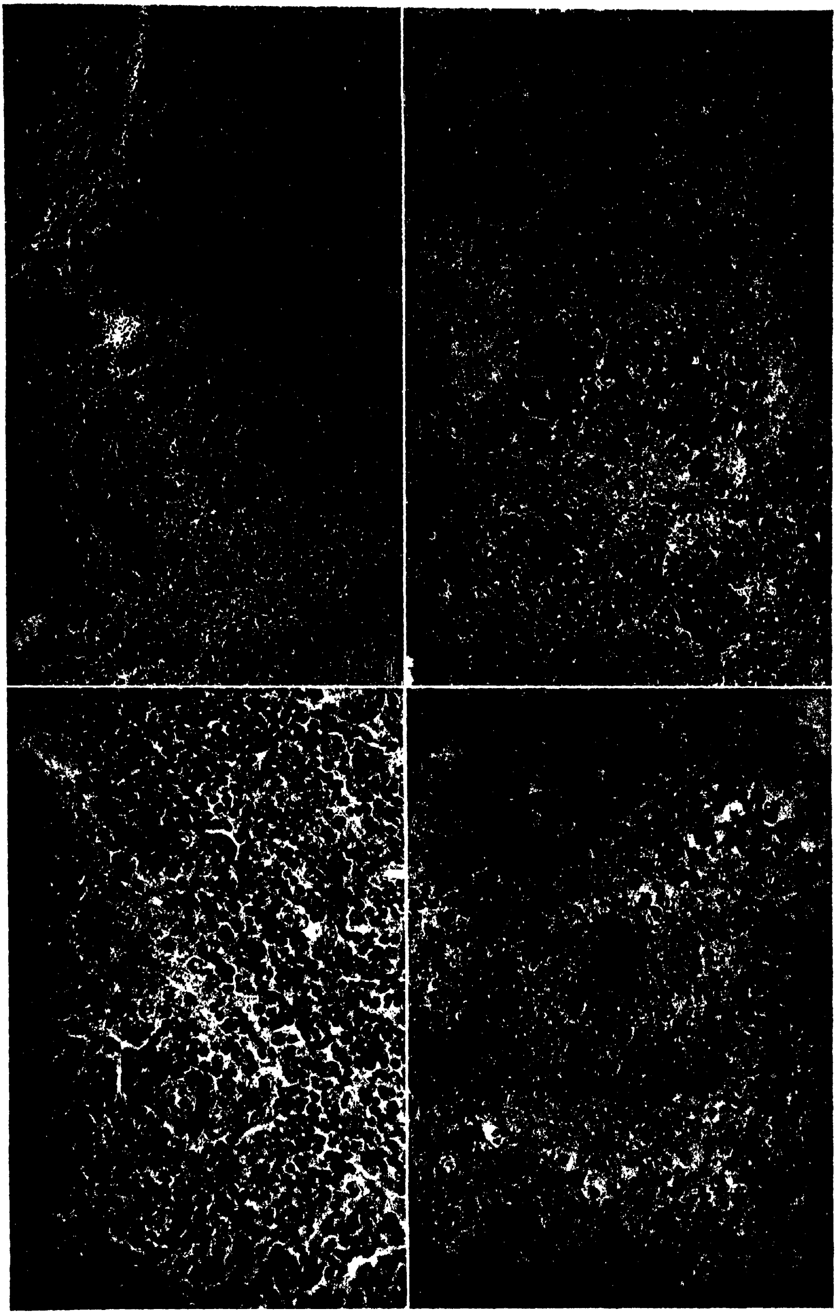
FIGS. 6 and 7. Early psittacosis lesions in liver; hyaline necrosis without much cellular infiltration. ×85 and 375. Eosin and methylene blue.

FIGS. 8 and 9. Lesions in liver caused by the virus of psittacosis; hyaline necrosis with infiltration of polymorphonuclear leucocytes. ×85 and 375. Eosin and methylene blue.

PLATE 10

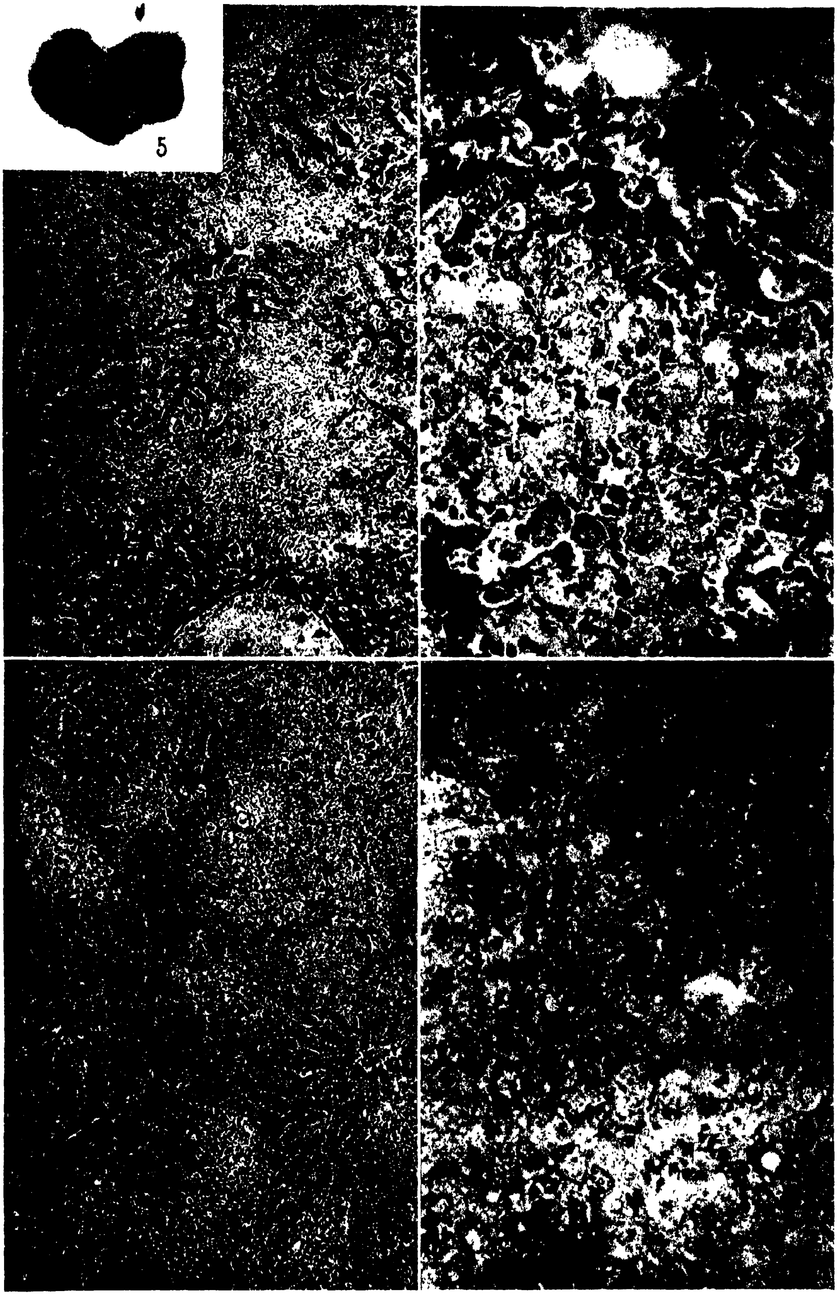
FIGS. 10 and 11. Liver lesions late in the course of a psittacosis infection; areas of hyaline necrosis replaced by nests of mononuclear cells. ×100 and 400. Eosin and methylene blue.

FIG. 12. Very extensive involvement of the liver that may appear early in the course of the infection; granular and fatty degeneration of the liver cells accompanied by nests of infiltrated cells. ×400. Eosin and methylene blue.



Photographed by Louis Schmidt

(Rivers and Berry: Psittacosis II)



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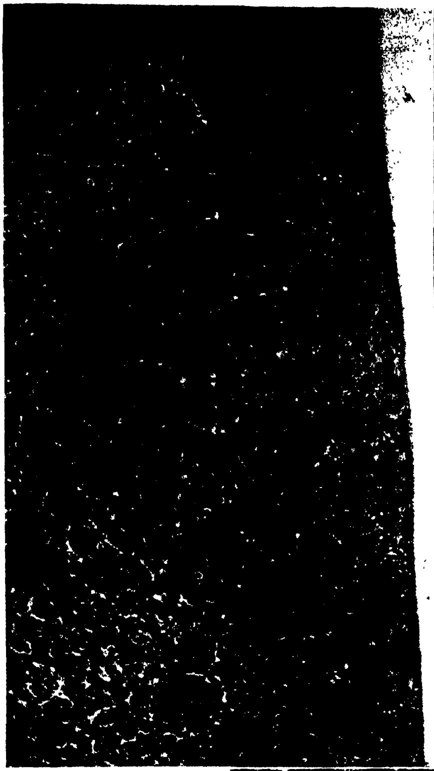

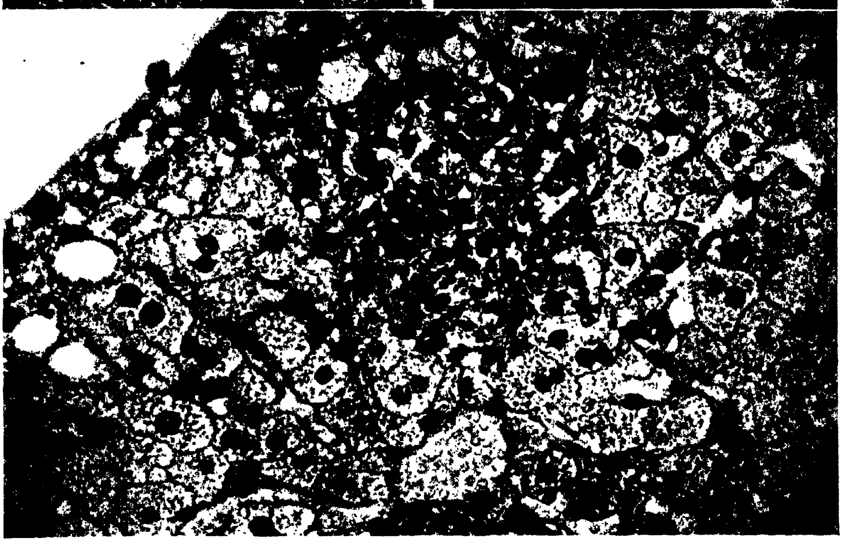

Photographed by Louis Schmidt

(Rivers and Berry: Psittacosis. II)

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PSITTACOSIS

III. EXPERIMENTALLY INDUCED INFECTIONS IN RABBITS AND GUINEA PIGS

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PLATE 11

(Received for publication, April 1, 1931)

When we began our investigations of psittacosis, the only animals known to be susceptible to the experimental disease were parrots, certain small birds closely related to parrots, and mice. Consequently it seemed desirable to ascertain whether other small laboratory animals are suitable for experiments with the disease. For this work, rabbits and guinea pigs were chosen. Results of the studies in which these animals were used and concerning which preliminary notes (1, 2) have already appeared are here set forth in detail.

Methods and Materials

Virus.—Virus from two sources was used; one strain came from an emulsion of the liver and spleen of Parrot N infected with virus originally obtained from a parrot with the natural disease, the other strain was derived from the liver and spleen of Parrot Wenz (WC) infected with material from a patient who had died of psittacosis. For more detailed information concerning these viruses, one may consult the first paper of this series.

Inoculations.—Intraperitoneal, intracutaneous, and corneal inoculations were used in a few instances. For the most part, however, the rabbits and guinea pigs were infected intracerebrally* and passages were accomplished by injections of 0.1–0.25 cc. of emulsified infectious brain tissue into the brains of normal rabbits and guinea pigs. The inoculations were made in the right half of the brain by means of a tuberculin syringe after the needle had been thrust through a small trephined opening in the skull. Transfers were made at the peak of the febrile reaction that usually came 3–7 days after inoculation.

* All operations were performed under ether anesthesia.

EXPERIMENTAL

Since symptoms and signs referable to the central nervous system constitute a conspicuous part of the clinical picture of psittacosis in man, and since many viruses are capable of propagation by means of brain to brain passages, it occurred to us that information concerning the etiological agent of psittacosis might be obtained by studying its effects on rabbits and guinea pigs infected intracerebrally. Furthermore, if it proved possible to establish the virus in these animals, an opportunity to investigate its distribution in their bodies would be provided. Moreover, we wished to determine whether passage of the virus through these more or less alien hosts would alter its pathogenicity for the natural host, the parrot, and for another alien host, the mouse. Finally, we desired to make certain investigations regarding immunity to psittacosis that would be facilitated in case the rabbit was found to be susceptible to the disease. Before discussing the results of our experiments, we shall give a general description of psittacosis experimentally induced by intracerebral inoculations of the virus in rabbits and guinea pigs. To this description, a few illustrative protocols are appended.

Clinical and Pathological Evidences of Infection

Following intracerebral inoculations of potent virus, all of the rabbits and guinea pigs, with a few exceptions, showed a high fever, *i.e.*, a temperature above 104°F. In many instances the temperature rose to 106–107°F, and occasionally reached 108.5°F. The peak of the febrile reaction was usually passed by the end of the 1st week after inoculation. During the febrile period, the animals appeared sick, refused to eat, lost weight extremely rapidly, and at times became ataxic. A few gravid pigs aborted. Some of the rabbits had convulsive seizures and died. As a rule, however, the animals recovered rapidly and appeared normal again 2 weeks after inoculation. The febrile reaction was so constant and striking that it was used as an index of infection.

The brains of rabbits and guinea pigs inoculated intracerebrally always showed pathological changes. The reaction was predominantly a meningitis characterized by infiltration of mononuclear and polymorphonuclear cells. The exudate was never extensive nor was it at any time purulent. The brain substance itself was not extensively damaged. Some degeneration of nerve cells and perivascular infiltration were seen. In the rabbits it was not always possible, because of the spontaneous encephalitis that occurs in them, to be certain of the cause of the changes around the vessels. In the pigs, however, there was no doubt that the virus of psittacosis produced perivascular infiltration.

The livers evidenced different degrees of enlargement and fatty degeneration. Some appeared to be almost normal, while others, particularly in the pigs, were so badly damaged that they had become chamois-colored and were very friable. In approximately 10 per cent of the animals, macroscopic evidences of necrosis or infarction (Figs. 1, 2) were found. Such necrotic or yellowish areas were occasionally surrounded by hemorrhagic zones. On section, no evidence of abscess formation was seen, and cultures of the tissues revealed no ordinary bacteria. Stained sections of the liver showed areas of necrosis somewhat similar to those seen in parrots and mice. There was no definite evidence of bile duct involvement. Occasionally vascular channels were thrombosed, but it was not possible to determine whether the vascular changes preceded or followed the necrosis. The splenic pulp was soft. The lungs, kidneys, and intestines were not particularly abnormal. Smears and sections of brains, livers, and spleens revealed none of the "minute bodies" found in parrots and mice infected with psittacosis.

Rabbit 2573, Mar. 28, 1930, was inoculated intracerebrally with 0.25 cc. of a brain emulsion from Rabbit 2563. *Mar. 29*, temp. 101.2°. *Mar. 30*, temp. 102.4°. *Mar. 31*, temp. 107°. *Apr. 1*, temp. 105.6°. *Apr. 2*, temp. 102.5°. *Apr. 3*, temp. 103.2°. *Apr. 4*, temp. 101.2°. The animal lost weight, but otherwise seemed in fair condition throughout the illness.

Rabbit 2610, Apr. 21, 1930, was inoculated intracerebrally with 0.25 cc. of a liver and spleen emulsion from Rabbit 2606. *Apr.22*, temp. 104.6°, animal excitable. *Apr. 23*, temp. 103.8°. *Apr. 24*, temp. 107.5°, rabbit very irritable. *Apr. 25*, temp. 106.2°, animal in fair condition, killed with chloroform and autopsied immediately. Brain injected and slightly edematous. Lungs normal in appearance; liver enlarged and friable; spleen slightly enlarged with soft pulp; kidneys badly pitted as the result of an old nephritis; intestines injected. Aerobic and anaerobic cultures of the brain, lungs, liver, and spleen remained sterile.

Guinea Pig 2605, Apr. 14, 1930, was inoculated intracerebrally with 0.1 cc. of a brain emulsion from Guinea Pig 2592. *Apr. 15*, temp. 101.5°. *Apr. 16*, temp. 103.2°. *Apr. 17*, temp. 107.2°. *Apr. 18*, temp. 106°. Except for loss of weight and fever, the animal gave very little clinical evidence of infection. Sacrificed and autopsied immediately. Brain injected and edematous; lungs normal; liver large and yellowish as though it had undergone fatty degeneration; splenic pulp soft. Aerobic and anaerobic cultures of brain, liver, and spleen revealed no ordinary bacteria.

Guinea Pig 2607, Apr. 18, 1930, was inoculated intracerebrally with 0.1 cc. of a brain emulsion from Guinea Pig 2605. *Apr. 19*, temp. 103.2°. *Apr. 20*, temp. 106.4°. *Apr. 21*, temp. 107°. *Apr. 22*, temp. 105.8°. Loss of weight and fever were the only clinical evidences of infection noted. Animal sacrificed and autopsied immediately. Brain injected and edematous; lungs normal; liver had undergone fatty degeneration and over its surface were seen yellowish areas that appeared to be the result of focal necrosis or infarction; splenic pulp soft; kidneys normal. Aerobic and anaerobic cultures of brain, lungs, liver, and spleen remained sterile.

From the general description of the clinical findings and from the illustrative protocols it is obvious that the chief reactions in rabbits and guinea pigs to intracerebral inoculations of psittacosis virus are loss of weight and fever which at times may be excessively high. Most animals reveal little or no clinical evidence of involvement of the brain and quickly make a complete recovery. Apart from the mild meningo-encephalitis that all of the intracerebrally infected animals show, the chief pathological changes are noted in the liver where striking lesions are found in approximately 10 per cent of the cases. These alterations are characterized by fatty degeneration, focal necrosis, and infarct-like processes (Figs. 1, 2).

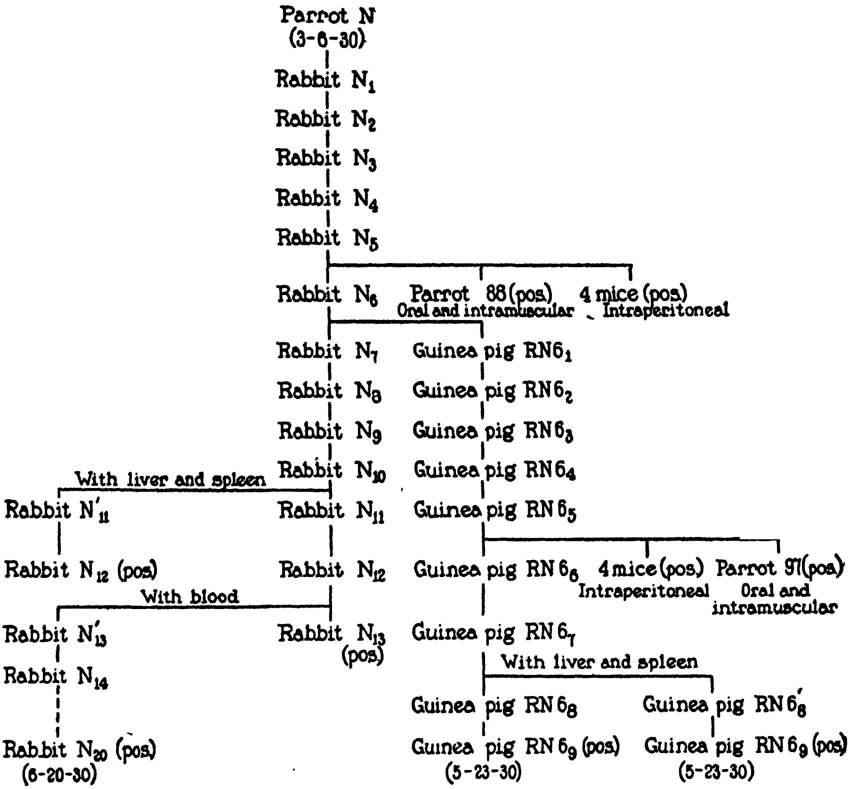
Results

Having detailed the clinical and pathological findings in rabbits and guinea pigs intracranially infected with psittacosis, we shall now describe the results of experiments on the serial passage of the virus in these animals.

Parrot N was infected by Krumwiede with a parrot strain of psittacosis virus and then transferred to The Rockefeller Institute. On Mar. 6, 1930, the bird died and at autopsy, a fatty liver, a large soft spleen, and a pericarditis were found. Cultures of the organs and pericardial exudate remained sterile, and on Mar. 8th 0.25 cc. of a 10 per cent liver and spleen emulsion were injected intracerebrally in a rabbit. The rabbit became sick and developed a high fever. On the 3rd day following inoculation, the animal was sacrificed and a 10 per cent brain emulsion was prepared and used to inoculate 2 normal rabbits intracranially. In this manner the virus was propagated serially through 5 groups of rabbits at which time, in addition to rabbits, a parrot and 4 mice were inoculated. All of these animals showed the clinical and pathological pictures of psittacosis. From a rabbit in the 10th set in the series, the virus was passed to normal rabbits by means of intracranial inoculations of emulsified liver and spleen. From a rabbit in the 12th set, blood was used to transfer the disease to a series of rabbits in which the virus was successfully propagated through 8 successive sets of animals.

From the 6th rabbit (Rabbit N_6) in the series of passages, 2 guinea pigs were inoculated (0.1 cc.) intracranially. From the brain of one of these pigs an emulsion was made and other pigs were infected intracerebrally in series. From a pig (Guinea Pig $RN6_5$) in the 5th set of the series, 4 mice and a parrot were inoculated and developed typical psittacosis. From the liver and spleen of a pig in the 7th set, an emulsion was made and injected (0.1 cc.) into the brain of 2 pigs. These animals became sick and developed fever. From them the virus was passed to other pigs.

Text-fig. 1 schematically portrays the serial passages of the parrot strain of psittacosis virus in rabbits and guinea pigs. Each set of animals consisted either of 2 rabbits or of 2 pigs. This fact, however, is not indicated in the diagram. Furthermore, when no notation concerning the inoculum and the route of inoculation occurs, it should be remembered that such passages were made from brain to brain.



TEXT-FIG. 1. Diagrammatic representation of experimental work conducted in rabbits and guinea pigs with a parrot strain of psittacosis virus. The mode of transfer, when undesignated, consisted of intracerebral inoculation of emulsified brain tissue.

Another series of passages in rabbits, similar to the one just described, was conducted with virus from the liver and spleen of Parrot Wenz C which had been infected with material of human origin. The active agent was passed intracerebrally through 14 successive sets of rabbits at which time the experiments were discontinued. From a rabbit in the 6th set in the series the virus was transferred

TABLE I

Summary of Reinoculation Experiments in Rabbits

Animal No.	1st inoculation Material Route, date	Virus in inoculum*	Strain of virus	Course after inoculation Highest T°	Time between inoculations	Reinoculation Material Route, date	Strain of virus	Course after inoculation Highest T°	Autopsy	Active immunity + or −
					days					
McG	Human blood i.p. 3–7–30	−		Negative	40	Intracerebral inoculation with rabbit brain in manner identical with control Rabbit 2606 4–16–30	P	108^2 Encephalitis, died	+	−
Old	Human blood i.p. 3–7–30	−		Negative	40		P	106^8 Encephalitis, killed on 8th day	+	−
2534	Rabbit testicle, liver, and spleen i.c. and i.d. 3–12–30	+	P	106^8 Tremulous and emaciated	35		P	Negative	Lived	+
					77	3rd inoculation Rabbit brain i.c. 5–28–30	P	Negative	Not done	+
Control 2606	Rabbit brain i.c. 4–16–30	+	P	108^5 Wild, tremulous, killed on 5th day		Autopsy showed typical psittacosis				
2562	Rabbit brain i.c. 3–24–30	+	H	104^8 Emaciated	23	Rabbit brain i.c. 4–16–30	P	104^4 Asymptomatic	Lived	±
2538	Parrot liver and spleen* i.t. and i.d. 3–12–30	+	H	No fever, orchitis	65	Intracerebral inoculation with rabbit brain in manner identical with control Rabbit 2678 5–28–30	P	Negative	−	+
					78		P	Negative	−	+
2552	Guinea pig brain i.c. 3–18–30	+	P	105^8 Tremulous	71		P	Negative	Not done	+

2574	Normal parrot liver and spleen i.c. and i.d. 3–29–30	–		Negative	60	Intracerebral inoculation with rabbit brain in manner identical with control Rabbit 2678 5–28–30	P	107^4 Tremulous	+	–
2575	Normal parrot liver and spleen i.c. and i.d. 3–29–30	–		Negative	60		P	107^3 Wild and tremulous	+	+
2577	Parrot liver and spleen i.c. and i.d. 3–29–30	+	P	104^6 Asymptomatic	60		P	106^8 Tremulous diarrhea	+	–
2579	Rabbit brain i.c. 3–31–30	+	H	Negative	58		P	107 Tremulous, diarrhea	Not done	–
2603	Rabbit brain i.c. 4–14–30	+	H	106^2 Asymptomatic	44		P	Negative	–	+
2622	Rabbit brain i.c. 4–24–30	+	P	106^8 Emaciated	34		P	Negative	–	+
2624	Rabbit brain i.c. 4–25–30	+	P	104^8 Tremulous	33		P	Negative	–	+
2628	Rabbit brain i.c. 4–26–30	+	H	106 Encephalitis	32		P	Negative	–	+
Control 2678	Rabbit brain i.c. 5–28–30	+	P	106^8 Tremulous, killed on 5th day		*	Autopsy showed typical psittacosis			

* Presence or absence of virus in inoculum determined in addition by other animal inoculations.

(intramuscularly and intraorally) to a parrot that sickened and died of psittacosis. From this bird, 2 other parrots were successfully infected by means of nasal secretions and feces respectively.

The work described above, some of which is schematically portrayed in Text-fig. 1, indicates that the virus of psittacosis is capable of indefinite propagation by means of brain to brain inoculations in rabbits and guinea pigs. Furthermore, it is evident that 5 such passages of the virus through rabbits, followed by 5 additional serial passages in guinea pigs, did not cause the active agent to lose its pathogenicity for parrots and mice. Moreover, the presence of the virus in rabbit's blood, liver, and spleen, and in guinea pig's liver and spleen was demonstrated. Finally, 2 strains of virus, one from a parrot and the other from man, affected rabbits and guinea pigs in a similar manner.

Immunity

Parrots upon recovery from an attack of psittacosis possess an active immunity (see first paper of this series), while mice may not (see second paper). It seemed important, therefore, to ascertain whether rabbits react to a primary infection with the virus by the development of an active immunity, and, if they do, to study by immunological methods the relation of the 2 strains of active agent (parrot and human) under investigation.

Fourteen rabbits that had received different materials intraperitoneally, intracerebrally, intradermally, and intratesticularly were reinoculated intracerebrally at varying periods of time, 23–78 days, following the first injections. Some of the primary inocula contained no virus, others had the parrot strain, while yet others carried the human strain. In each instance, the parrot strain was used for reinoculation. Of the 6 rabbits (Table I) that received no virus at the time of the first inoculation, all developed high fever and were sick following reinoculation with the virus. Of the 8 rabbits, however, that received virus in the primary inoculum, only 1 showed fever. This animal was first reinoculated 23 days after its primary infection. In contrast, 65 days after receiving its original dose of virus, it was reinoculated a second time and was found to be fully resistant.

The results of the reinoculation experiments summarized in Table I clearly show that rabbits upon recovery from psittacosis are actively immune. Furthermore, it is evident that the 2 strains of virus under investigation are immunologically similar.

Since the rabbit responds to intracerebral inoculations of the virus with a striking rise of temperature, it occurred to us that its febrile reaction might be used as an index of infection, thus indicating the state of activity of virus in neutralization tests. Examination in which the virus serum mixtures were tested in the brains of rabbits are described below.

Serum was collected from a patient on the 2nd day of the disease and also on the 10th day after subsidence of fever. A normal individual who had never had psittacosis was bled for control serum. Brain emulsion containing virus was diluted 1:5, 1:10, 1:20. Portions of these dilutions were mixed with equal amounts respectively of the 3 specimens of serum. The mixtures were allowed to stand 1 hour at 37°C. and then 0.25 cc. of each were injected intracerebrally in a rabbit. Thus 9 rabbits were inoculated. In one instance only, neutralization of the virus by convalescent serum appeared to have occurred. When the experiment was repeated, however, no evidence of inactivation of the virus by convalescent serum was obtained.

From the results of the neutralization experiments described above, one is not justified in concluding that convalescent human serum shows any appreciable amount of neutralizing power. These findings are similar to those obtained when mice (see second paper of this series) are employed as the test animal.

DISCUSSION

We have reported the results of our work dealing with the propagation of the virus of psittacosis in the brains of rabbits and guinea pigs. Other workers (3, 4) have confirmed our observations, and, in addition, have been able to obtain similar results by means of intratesticular inoculations. Furthermore, they have shown that the skin of rabbits and guinea pigs is suceptible to the virus and can be used for titrations of its activity. Moreover, the failure of human convalescent serum to inactivate virus has been observed by other workers (4, 5) as well as by ourselves. Thus, no experimental proof has been adduced to show that convalescent serum is of value in the treatment of psittacosis.

CONCLUSIONS

1. Rabbits and guinea pigs are susceptible to psittacosis virus introduced intracerebrally. By means of brain to brain passages in these animals the active agent is capable of propagation indefinitely.

2. Serial passages of the virus through rabbits and guinea pigs do not cause the active agent to lose its pathogenicity for parrots and mice.

3. The chief clinical evidences of infection in rabbits and guinea pigs following intracranial inoculation of the virus are fever and loss of weight. The pathological changes are characterized by a mild meningo-encephalitis, and fatty degeneration, focal necrosis, and infarction of the liver.

4. Rabbits upon recovery from an attack of psittacosis are actively immune.

5. Two strains of virus, human and parrot, were found to be immunologically similar.

6. No evidence was obtained to show that human convalescent serum possesses an appreciable amount of neutralizing substances.

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EXPLANATION OF PLATE 11

FIG. 1. Liver of a guinea pig, infected with psittacosis, showing fatty degeneration and areas of necrosis or infarction. × 1.

FIG. 2. Liver of a rabbit, infected with psittacosis, showing areas of necrosis or infarction. ×1.

1

2

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PSITTACOSIS

IV. EXPERIMENTALLY INDUCED INFECTIONS IN MONKEYS

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PLATES 12 TO 17

(Received for publication, April 1, 1931)

In the three papers immediately preceding this one, psittacosis experimentally induced in parrots, rabbits, guinea pigs, and mice was described. None of the animals employed in the work detailed, however, evidenced pulmonary signs or lesions of any significance. Inasmuch as psittacosis in man manifests itself chiefly by pathological changes in the lungs, and since no one had employed monkeys for the experimental study of the malady, we decided to determine whether it is possible to produce in certain lower primates pulmonary lesions similar to those found in human beings infected with the virus of psittacosis. In this study, 6 experiments were performed in which 12 monkeys were inoculated one or more times.

Methods and Materials

Virus.—The psittacosis virus was obtained from the livers and spleens of Wenz C mice (see second paper of this series) carrying a human strain of the active agent. The emulsions containing the virus for each experiment were shown to be free from ordinary aerobic and anaerobic bacteria.

Animals.—Healthy medium sized Indian monkeys (*Macacus rhesus*) proved to be satisfactory for the work. Only animals whose lungs were shown by X-ray examination to be normal were used.

*Inoculations.**—The majority of monkeys was infected by intratracheal inoculations (1–4 cc.) accomplished by thrusting a small needle into the trachea below the larynx and then injecting the emulsion with a syringe. 2 monkeys were inoculated intracerebrally (1 cc.) through a small trephined opening in the skull. 2 monkeys received the infectious agent by intranasal instillations (1–2 cc.) of organ emulsions.

* All operations were performed under ether anesthesia.

EXPERIMENTAL

The objects of the first experiment were to determine whether monkeys are susceptible to the virus of psittacosis injected intratracheally or intracerebrally, and to ascertain whether the virus can be propagated by serial passages in these animals.

Experiment I

Monkey A, May 20: Temp. before inoculation not taken. Received intratracheally 2.5 cc. of liver and spleen emulsion from mice WC_{17}. *May 22*, temp. 103.8°, appears normal. *May 23*, temp. 105.4°, condition same. *May 24*, temp. 104.8°. *May 25*, temp. 104°. *May 26*, temp. 105°, seems sick and eating poorly. X-ray of chest shows a shadow extending to left of heart and mottling of both lower lobes behind the diaphragm. *May 27*, temp. 103.4°, still appears sick. *May 28*, temp. 102.6°, condition improved. X-ray of chest reveals an extension of the shadows noted on the 26th. Animal killed with chloroform. *Autopsy*: Heart, pericardium, liver, spleen, and kidneys appear normal. Lungs: No pleurisy. Both lower lobes are partially consolidated and have taken on a lilac-pink color. The other lobes on the right are involved in a patchy manner. The consolidated lobes on section appear homogeneous, smooth, free of edema. The bronchi are not raised above the cut surface and contain no exudate. The hilar lymph glands are enlarged and contain a few small hemorrhages. Smears from the lungs showed no ordinary bacteria, and none of the "minute bodies" found in parrots and mice infected with psittacosis virus. Cultures, aerobic and anaerobic, of the lungs and liver remained sterile. Pieces of involved lung were emulsified and injected into 4 mice intraperitoneally and into Monkey E intratracheally. 2 of the mice died 6 days after inoculation and showed typical psittacosis lesions in the liver and spleen. Numerous "minute bodies" were found in smears from these mouse organs. The other 2 mice were sick for a number of days and finally died of psittacosis.

Monkey E, May 29: Temp. before inoculation, 101.8°. X-ray of chest negative. Received intratracheally 4 cc. of lung emulsion from Monkey A. *May 30*, temp. 102°, animal well. *May 31*, temp. 104°, seems sick. *June 1*, temp. 105°, sick. *June 2*, temp. 103.6°, sick and has diarrhea. X-ray of chest shows no obvious lesions. *June 3*, temp. 103°. *June 4*, temp. 105.8°, diarrhea persists. X-ray of chest negative. *June 5*, temp. 104.4°. *June 6*, temp. 102.5°, animal still has diarrhea. X-ray of chest negative. Killed with chloroform and autopsied immediately. Pericardium contains 1 cc. of sticky greyish exudate; no "minute bodies" or bacteria found in smears; cultures sterile. Right lung bound down by fresh fibrinous adhesions. 3 upper lobes normal. Right lower lobe shows scattered subpleural hemorrhages. On section several small greyish nodules noted in the parenchyma. Hilar lymph nodes enlarged and hemorrhagic. Liver has a small yellowish area near the point where the round ligament emerges. Cultures from

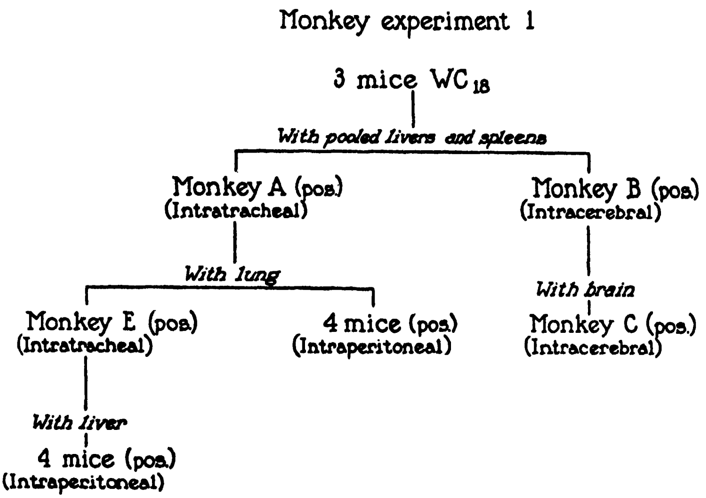
the lungs and liver sterile; no "minute bodies" found in smears from these organs. An emulsion from the liver was injected into 4 mice, 2 of which died 9 days later. The other 2 died 17 and 30 days respectively after inoculation. All the mice had psittacosis.

Monkey B, May 20: Received in the brain 0.75 cc. of an emulsion similar to that given Monkey A. *May 22*, temp. 104°, animal sick, weak, and has diarrhea. Was observed during a convulsion. *May 23*, temp. 104.5°, weak, ataxic; severe diarrhea. *May 24*, temp. 105°, condition worse. *May 25*, temp. 103.8°. Animal weaker and more ataxic. *May 26*, temp. 99°. Unconscious and having repeated convulsive seizures. Killed with chloroform and autopsied immediately. Lungs, liver, and spleen are negative. Brain injected and edematous. Cultures from brain and other organs sterile. Smears negative for the "minute bodies." Stained sections of the brain showed a mild encephalitis with some degeneration of nerve cells. The predominant lesion was a meningeal reaction characterized by mononuclear infiltration.

Monkey C, May 28: Received intracerebrally 0.75 cc. of a brain emulsion from Monkey B. *May 29*, temp. 102.8°. Animal well. *May 30*, temp. 102.6°. *May 31*, temp. 103°. *June 1*, temp. 102.6°. Animal still seems well. *June 2*, temp. 105°. *June 3–7*, temp. 104° or above. Animal definitely sick. X-ray of chest taken on June 4 negative. *June 8–14*, temp. 102–102.6°. Monkey appears normal again.

The results of Experiment I detailed in the protocols above and portrayed in Text-fig. 1 indicate that macaques are susceptible to the virus of psittacosis and that the virus can be passed from monkey to monkey by intratracheal or by intracerebral inoculations. There are several points of interest, however, that should be noted. The reactions in the first monkeys of the 2 series were much more severe than were those in the second lot of animals. Moreover, mice inoculated with emulsions of Monkey A's lung and Monkey E's liver developed psittacosis, but they died more slowly than did mice inoculated with mouse passage virus. These facts suggest the possibility that passage of the virus through monkeys alters it in such a manner that transfers from monkey to monkey become relatively difficult. Similar conditions may hold for human beings who seem to be quite susceptible to the virus emanating from parrots, but relatively non-susceptible when exposed to the disease in man. Attention should also be directed to the fact that monkeys receiving the virus intracerebrally developed no pulmonary lesions. Thus, it appears that the portal of entry of the virus profoundly influences its localization and the pathological changes

caused by it in these animals. This fact lends evidence in favor of the idea that the active agent enters man, in whom involvement of the lungs usually occurs, through the upper respiratory tract. Finally, the "minute bodies" found in parrots and mice infected with psittacosis virus were not seen in the lungs and brains of monkeys attacked by the same active agent. Yet emulsions of these organs produced psittacosis in mice, and smears from their livers and spleens showed the small bodies. Failure to find these structures in monkeys, however, does not necessarily mean that none were present.



TEXT-FIG. 1. Diagrammatic representation of Experiment I

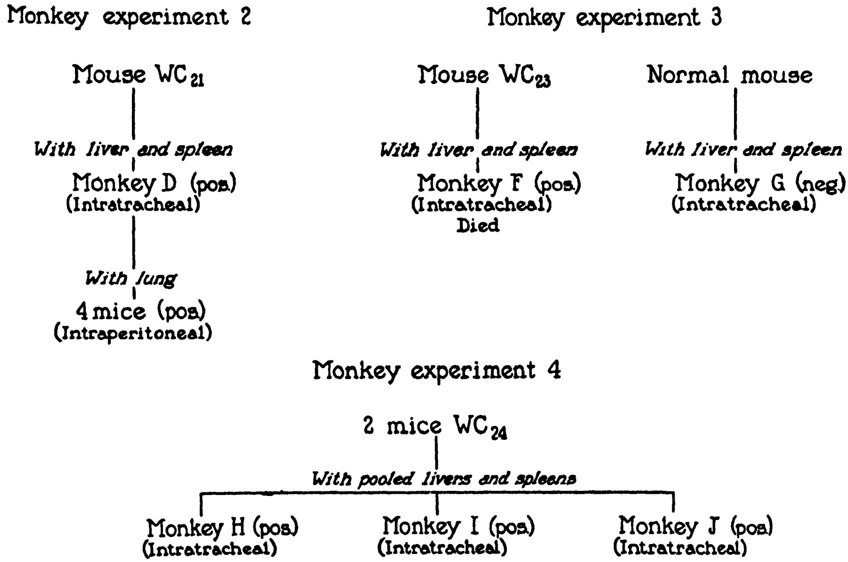
Before proceeding it seemed advisable to repeat part of the first experiment for confirmatory purposes.

Experiment II

Monkey D, May 28: Temp. 102.4°. X-ray of chest negative (Fig. 11). *May 29*, animal received intratracheally 4 cc. of pooled liver and spleen emulsion from mice WC_{21}. *May 30*, temp. 103.8°, seems well. *May 31*, temp. 105°, eating poorly. *June 1*, temp. 104.5°, sick. *June 2*, temp. 104.8°, slight cough. X-ray of chest reveals involvement of a large part of the right lung and of the left lower lobe (Fig. 12). Killed with chloroform and autopsied immediately. No evidence of pleurisy. The 4 lobes of the right lung and the two lower lobes of the left reveal a hemorrhagic, purplish consolidation spreading out from the hilum. On section, the surface is smooth and dry, similar to the cut surface of meat. No exudate in

bronchi. Free, straw-colored fluid in pericardium. Liver, spleen, intestines, adrenals, and kidneys appear normal. In smears from the lungs and pericardial fluid no "minute bodies" were found. Cultures of lungs and liver sterile. 4 mice were inoculated (0.5 cc. each) intraperitoneally with a lung emulsion from Monkey D. All died of psittacosis; 1 on the 3rd, 1 on the 6th, 1 on the 15th, and 1 on the 18th day respectively after inoculation.

The results of Experiment II, shown in Text-fig. 2, confirm those obtained in the first experiment. Having determined that liver and spleen emulsions from mice infected with psittacosis cause a consolida-



TEXT-FIG. 2. Diagrammatic representation of Experiments II, III, and IV

tion of the lungs, we decided to see what effect similar emulsions from normal mice would have. This was undertaken in Experiment III.

Experiment III

Monkey F received intratracheally 2.5 cc. of an emulsion of pooled livers and spleens from psittacosis mice WC_{23}, while Monkey G received in a similar manner 2.5 cc. of an emulsion of pooled livers and spleens from normal mice.

Monkey F, June 2: Temp. 101.6°. X-ray of chest negative. *June 3*, date of inoculation. *June 4*, temp. 106.2°, seems well. *June 5*, temp. 105.2°, stools loose. *June 6*, temp. 104.6°, sick, has cough. X-ray of chest shows extensive shadows in both lower lobes behind the dome of the diaphragm and also in the

right middle and upper lobes. *June 7*, temp. 104°, considerable difficulty in breathing. X-ray indicates that the middle lobe on the left side is also involved. *June 8*, temp. 104.8°, very sick, does not eat, has difficulty in breathing, stools loose. *June 9*, temp. 102.2°. X-ray shows large shadows throughout the right side with extension of the pneumonia in left lower and middle lobes; the left upper only remains clear. Shortly after the X-ray was taken the monkey died and was autopsied immediately. Lungs: No pleurisy; many subpleural hemorrhages. Right: The upper lobe with the exception of the edges is completely consolidated. The two middle lobes are less completely involved, the edges being quite free. The lower lobe is almost completely consolidated. Left: Upper and middle lobes are only slightly involved. Lower lobe is almost completely consolidated, edges alone remaining free. On section the consolidated lobes do not have the appearance usually seen in ordinary lobar pneumonia. They are not granular, have a meaty appearance with slight peribronchial areas of pallor. No exudate is noted in the bronchi. Heart: 3 or 4 cc. of clear straw-colored fluid in pericardium. Liver: Enlarged, edges rounded. Near where the round ligament emerges there appears to be a small infarct. The liver has a peculiar appearance suggesting widespread fatty degeneration with areas of necrosis. Spleen: Pulp very soft. Adrenals and kidneys appear normal. Cultures of the lungs, liver, and pericardial fluid sterile. Smears for the "minute bodies" negative.

Monkey G was treated in a manner similar to that in which F was handled, with the exception that an emulsion of normal spleens and livers was used as an inoculum. The animal evidenced no signs of illness, never had any fever, and 4 X-rays of the chest revealed no areas of consolidation.

The results of the above experiment (Text-fig. 2) indicate that an emulsion of livers and spleens from normal mice introduced intratracheally does not produce a pneumonia in monkeys. In the next experiment, No. IV, 3 monkeys received intratracheally psittacosis virus in an emulsion (2 cc. to each animal) of pooled livers and spleens from mice WC_{24}. The animals, having been watched and examined frequently by means of the X-ray were killed 2, 6, and 13 days respectively after inoculation. In this way lungs were obtained during different stages of the disease in order to study the evolution of the pathological process.

Experiment IV

Monkey H, June 3: Temp. 103.5°. X-ray of chest negative. *June 5*, temp. 103.2°. *June 6*, temp. 102.4°. *June 7*, date of intratracheal inoculation. *June 8*, temp. 105.3°, diarrhea. *June 9*, temp. 104.8°, diarrhea persists. X-ray of chest shows extensive mottling of right lower lobe with smaller shadows in the left lower. There is evidence of spreading of the infection along the bronchial tree into the

upper and middle lobes (Fig. 1). Animal killed with chloroform and autopsied immediately. Pericardium, heart, spleen, kidneys, and adrenals seem to be normal. A small whitish area in the edge of one lobe of liver near the round ligament. Lungs show lesions spreading out from the hilum along the bronchial tree into all the lobes. The involved portions are purplish pink, only partially consolidated, but sharply demarcated from the surrounding normal lung tissue. Cultures of the lungs sterile.

Monkey I, June 3: Temp. 102.5°. X-ray of chest negative (Fig. 5). *June 5*, temp. 103°. *June 6*, temp. 102.4°. *June 7*, date of intratracheal inoculation. *June 8*, temp. 105°, eating poorly. *June 9*, temp. 103.5°, sick and weak. X-ray shows small shadows in both lower lobes near the hilum (Fig. 6). *June 10*, temp. 103.2°, condition unchanged. X-ray: Shadows in both lower lobes have extended toward the periphery. The 2 middle lobes and possibly the upper on the right side are consolidated near the hilum. *June 11*, temp. 104.4°, condition worse. X-ray: The areas of consolidation have increased in size and the shadows are more intense. *June 12*, temp. 104.8°, condition worse. X-ray: Shadows indicate that the areas of consolidation have almost reached the periphery (Fig. 7). *June 13*, temp. 103.5°. X-ray shows no change. *June 14*, temp. 105°, still quite sick and has lost weight. *June 15*, temp. 104.2°. *June 16*, temp. 103.4°, better and eating a small amount of food. X-ray: Some clearing of the shadows at the periphery of the lesions (Fig. 8). *June 18*, temp. 100.8°, much better. X-ray: Marked clearing of the shadows (Fig. 9). *June 20*, temp. 102.4°, animal seems almost normal again. X-ray: A few irregular shadows persist near the hilum (Fig. 10). Monkey killed and autopsied immediately. Heart and pericardium normal. Both pleural cavities clear. Lungs: The upper and 2 middle lobes on the right bound to each other by loose adhesions. Only slight changes near the hilum are noted in the upper and one of the middle lobes, while in the other middle and lower lobes quite firm areas are still detected near the hilum around the vessels and bronchi. On the left side, the upper lobe is normal, while near the hilum areas of consolidation in the middle and lower lobes still exist. These are less extensive than are those in the right lower. On section the involved lobes exhibit air-containing alveoli around the periphery, while in the central portions along the bronchi and vessels a yellowish gray semiconsolidated tissue, dry and relatively smooth appearing, is found. Hilar lymph glands not greatly enlarged. Liver shows fatty degeneration. Kidneys and spleen normal in appearance. Hemorrhage in medulla of right adrenal gland. Cultures of lungs: One remained sterile, the other showed a few indifferent streptococci.

Monkey J, June 5: Temp. 104.3°. X-ray of chest negative. *June 7*, date of intratracheal inoculation. *June 8*, temp. 105°, seems well. *June 9*, temp. 104.5°, condition unchanged. X-ray: Shadows in both lower lobes near the hilum. *June 10*, temp. 104.2°, sick, weak, not eating. X-ray: All lobes on the right seem to be involved near the hilum; extension of shadow in left lower lobe. *June 11*, temp. 105.6°, worse. X-ray: Increase of shadows on both sides, but left upper and

middle lobes still relatively clear. *June 12*, temp. 104.6°, condition same. X-ray: No change in shadows. *June 13*, temp. 103.2°, animal still very sick and has difficulty in breathing. X-ray: The right lung is almost completely consolidated, the left upper and middle lobes remain relatively clear (Fig. 4). Animal killed with chloroform and autopsied immediately. The lobes on the right side, bound to each other and to the chest wall by a sticky mucoid fibrinous exudate are completely consolidated with the exception of the peripheral portions. Slight amount of pleurisy on the left side with extensive consolidation of the lower lobe, the other 2 lobes relatively free. On section the color and consistency of the lungs similar to that described in other monkeys. Hilar lymph glands enlarged. Heart enlarged. In the pericardial sac are 1 or 2 cc. of sticky, whitish exudate. Liver shows a small area of necrosis near the round ligament. The intestines, spleen, kidneys, adrenal glands, and brain appear normal. In the different exudate and organs, no bacteria or "minute bodies" were found, and cultures from them remained sterile.

In Experiment IV, by means of X-ray examinations that were confirmed by autopsy findings, it was possible to follow the spread of the consolidation from the hilum towards the periphery and to see it regress in the reverse order. The course of events, shown by this set of photographs to occur in monkeys infected with psittacosis virus, parallels that observed in the lungs of man infected with the same active agent (1). Having demonstrated conclusively that a pneumonia in monkeys regularly follows the intratracheal inoculation of psittacosis virus, we considered it essential to ascertain, Experiment V, whether a pulmonary infection occurs when the inciting agent is instilled intranasally.

Experiment V

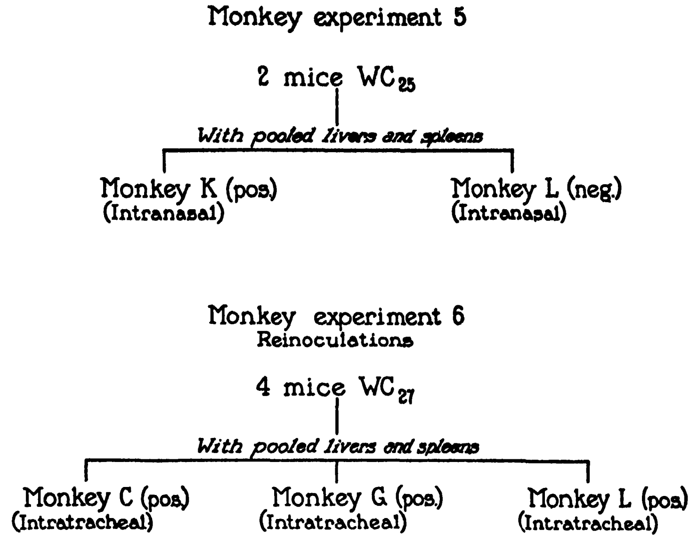
Into the noses of Monkeys K and L respectively, were instilled 1.5 cc. of an emulsion of pooled livers and spleens from psittacosis mice WC_{25}.

Monkey K, June 10: Temp. 101.6°. X-ray of chest negative (Fig. 2). *June 11*, date of intranasal inoculation. *June 12*, temp. 102.8°. *June 13*, temp. 105.4°, animal well. X-ray: Chest still clear. *June 14*, temp. 105°. X-ray: Beginning shadow at hilum on the right side, and slight mottling of right lower lobe. *June 15*, temp. 105.6°, eating poorly, slight diarrhea. *June 16*, temp. 105°, less diarrhea. X-ray: Shadow at hilum has increased and is spreading in a manner similar to that observed in man (Fig. 3). *June 17*, temp. 104.2°, animal does not appear very sick. X-ray shadows more intense. *June 18*, temp. 104.6°, condition same. X-ray shadows unchanged. Animal killed with chloroform and autopsied immediately. All organs appear normal with the exception of the right lung. In the lower portion of the upper lobe and at the top of the lower lobe are areas of

consolidation surrounding the main bronchi. On section, the surface of the consolidated tissue is dry, gray, and smooth. Cultures sterile.

Monkey L received an inoculum similar to that of Monkey K, but at no time did it have fever, or pulmonary consolidation as evidenced by repeated X-ray photographs.

Of the 2 monkeys receiving intranasal inoculations of psittacosis virus as described in the above experiment, 1 developed a typical pneumonia with fever while the other showed no signs of infection. The fact that only 1 animal became sick after this type of inoculation



TEXT-FIG. 3. Diagrammatic representation of Experiments V and VI

indicates that intranasal inoculations with psittacosis virus will not infect monkeys as regularly as do intratracheal injections. Nevertheless, it shows that, when the virus does enter in such a manner a pneumonia similar to that seen in man occurs. Thus, experimental evidence has been adduced in favor of the idea that man usually becomes infected through the upper respiratory tract.

The last experiment (No. VI, see Text-fig. 3), in which monkeys were used, consisted of reinoculations of Monkeys C, G, and L. Monkey C had recovered from an intracerebral inoculation administered 26 days previously; Monkey G had been given an intratracheal injection

of an emulsion of livers and spleens from normal mice; Monkey L had had virus instilled in the nose 12 days previously without any evidence of infection. Each animal was reinoculated intratracheally with 1 cc. of mouse virus, and all responded with a moderately severe pneumonia that was verified by X-ray examinations and autopsies. Monkey C was the only animal in the group that had previously evidenced signs of infection due to psittacosis virus. Consequently, it was the only one that might have been expected to show an immunity to reinfection. It did not, however. Whether this lack of immunity was due to the type of the first infection (intracerebral), whether 26 days between the first and second inoculations was too short a period of time for a demonstrable immunity to appear, or whether monkeys do not develop a solid immunity against psittacosis virus are questions that cannot be answered at present.

Microscopic Pathology

In the three preceding papers the pathological changes induced by psittacosis virus in parrots, mice, rabbits, and guinea pigs were described. Significant lesions, however, were not found in the lungs of these animals. Moreover, pulmonary lesions experimentally induced by the action of the virus in such hosts have been described by only a few workers, and in each instance the picture presented leaves one in doubt as to whether or not the morbid changes were caused by the active agent under discussion. In the present communication, the clinical and gross pathological pictures of psittacosis pneumonia in monkeys have been recorded. It now remains to describe the microscopic pathology of the pulmonary changes. Inasmuch as the animals were sacrificed after different intervals of time (2–13 days) had elapsed following inoculation, an excellent opportunity for a study of all stages of the morbid conditions in the lungs was presented. Therefore, the monkeys will be discussed in the order in which they were killed. There is, however, at least one fallacy in this mode of presentation, *viz.*, after an infection in the lungs has been under way for several days, early and advanced lesions may be found in the same animal. This fact must be borne in mind.

Two Days after Inoculation.—Monkey H. Examination of sections of whole lobes reveals that the consolidation first appears around the vessels and bronchi

near the hilum. Extending from the small areas of consolidation the walls of the alveoli (Fig. 15) are thickened and edematous, and show evidence of cellular infiltration. Study of many such sections leads one to suspect that the infection spreads along the alveolar walls. In many areas these structures are engorged with blood and contain a few polymorphonuclear cells (Fig. 16). In other places they are thickened by the swelling of alveolar cells and the infiltration of mononuclear elements (Fig. 17). Serous exudate (Fig. 16), fibrin, and desquamated cells are seen in many alveoli, while in definitely consolidated areas some of the alveolar spaces are filled with cells predominantly of the polymorphonuclear type (Fig. 18).

Four Days after Inoculation.—Monkey D. Sections of entire lobes again show that the consolidation begins near the hilum and spreads peripherally. In Fig. 20, the alveoli near the pleura are distended with serous exudate in which are embedded a few cells. In more centrally located areas, the number of cells in the exudate increases, and the alveolar walls are thickened (Fig. 21). As the hilum is approached, completely consolidated lung is encountered, in which some of the alveolar walls appear necrotic and the alveolar spaces are filled with a mixture of polymorphonuclear and large mononuclear cells (Fig. 22), while other areas reveal thickened alveolar walls and alveoli distended entirely by mononuclear cells (Fig. 24). Small hemorrhages and masses of fibrin are scattered throughout the involved portions of the lung. The origin of the mononuclear cells—there seems to be more than one kind present (Fig. 23)—in the exudate is not definitely known.

Six Days after Inoculation.—Monkeys F and J. The pictures presented by these monkeys are similar to those found in sections of Monkey D. Some differences, however, may be noted, for example, the infrequency of polymorphonuclear cells, and the appearance in the alveolar walls in certain places of giant cells and mitotic figures.

Seven Days after Inoculation.—Monkeys K and L. At this stage of the infection, practically no polymorphonuclear cells are found, hemorrhages are rarely seen, fibrin and serous exudates are still present in places, and a distinct perivascular cuffing with mononuclear cells, many of which are distended with pigmented granules, has made its appearance. This phenomenon of cuffing is noted in earlier monkeys but not to such an extent as in F and J and the others to follow. Evidences of cellular proliferation in the alveolar walls are pronounced and in many places it is difficult to distinguish these structures from the exudate or cells in the alveolar spaces (Fig. 19—Monkey K inoculated intranasally).

Eight Days after Inoculation.—Monkey A. This animal was killed on the 8th day after inoculation, the 1st day that an improvement in its condition was noted. An attempt to describe the microscopic pathology observed in the lungs will not be made, since no description can equal the pictures presented in Figs. 25, 26, and 27, an examination of which will show the condition of the alveolar walls and the contents of the alveolar spaces, and justify the conclusion that the pathological changes present are not those usually encountered in lobar pneumonia of man and monkeys.

Ten and Twelve Days after Inoculation.—Monkeys C and G. Clinically these animals were recovering from their pneumonia. Examination of the sections reveal evidences of resolution that will be discussed in connection with the next animal (Monkey I).

Thirteen Days after Inoculation.—Monkey I. This animal was killed 13 days after inoculation, 4 days after it began to show clinical evidences of improvement, and at a time when the X-ray photographs had almost returned to normal (Figs. 5–10). Grossly the lungs reveal more evidences of consolidation than had been expected from the X-ray pictures. Examinations of stained sections of whole lobes confirm the macroscopic pathology. Areas of dense tissue (Fig. 28) in which it is difficult to distinguish alveolar walls from alveolar contents are seen. Such areas are separated from each other by large air-containing spaces or distended alveoli (Fig. 29). A superficial examination of sections stained with eosin methylene blue suggests that a great deal of organization is taking place; but by the use of Mallory's aniline blue and orange G stain (Figs. 29 and 30) it becomes evident that only a slight amount of connective tissue is being laid down and that the alveoli are filled with large mononuclear cells. It is also obvious that resolution is occurring from the periphery towards the hilum. Consequently, the most marked involvement of the tissues is still found around the large bronchi and vessels near the hilum. Perivascular cuffing, in which lymphocytes are numerous, is conspicuous.

"Minute Bodies."—In stained sections of the consolidated lungs from monkeys, the "minute bodies" found in livers, spleens, etc., of parrots and mice infected with the virus of psittacosis were searched for diligently. None were seen. Failure to find them, however, does not necessarily indicate that they were not present in small numbers. Many cells in the aveoli, alveolar walls, and perivascular cuffs contained granules of different sizes and nature. None of these however, resembled the "minute bodies."

Psittacosis Pneumonia in Rabbits

After we recognized the fact that intracerebral inoculations of the virus do not induce pneumonia in monkeys, while intratracheal and intranasal injections of the same agent do, we decided, in spite of our previous negative results with intracerebral methods, to infect some rabbits intratracheally in order to ascertain whether a pneumonia can be produced in these animals by such a procedure.

2 groups of rabbits were used. One set of animals received intratracheally 1 cc. each of an emulsion of livers and spleens from mice infected with psittacosis virus, the rabbits of the other group were similarly inoculated with an emulsion of livers and spleens from normal mice. The animals that received the infectious material developed fever. Some of them died, while others were sacrificed at different intervals for bacteriological and pathological studies. Cultures of the lungs for the

presence of aerobic and anaerobic bacteria remained sterile. Macroscopically and microscopically the lungs showed a pneumonia similar to that observed in monkeys 2–4 days after intratracheal injections of the virus. The rabbits that received the emulsions of normal livers and spleens had no fever and, when they were sacrificed for examination of their lungs, revealed no pneumonia.

From the results of the above experiment it is obvious that rabbits develop pulmonary lesions following intratracheal injections of psittacosis virus. Experiments reported in the third paper of this series, however, show conclusively that such lesions do not occur, or only rarely, when the infectious agent is placed intracerebrally.

DISCUSSION

The evidence—clinical findings, X-ray photographs, gross and microscopic pathology, and bacteriological observations—justifies the conclusion that emulsions of livers and spleens from psittacosis-infected mice injected intratracheally or instilled intranasally in monkeys are capable of producing a pneumonia unassociated with ordinary bacteria. Moreover, such a pneumonia is similar to, if not entirely identical with that observed in man (1–4) infected with the virus of psittacosis. Furthermore, the experimental study of the disease in monkeys offers opportunities for observation that cannot be made in man, because the animals can be sacrificed at any time during the course of the malady and in them pictures uncomplicated by secondary bacterial invasion are the rule rather than the exception.

The pneumonia occurring in monkeys under experimental conditions begins around the large bronchi and vessels near the hilum and apparently spreads towards the periphery along the alveolar walls. Resolution occurs in the reverse order. The pleura is rarely involved. Many of the evidences of pulmonary involvement—vascular engorgement, cellular infiltration, necrosis of alveolar walls, hemorrhage, serous exudation, fibrin deposition, desquamation of alveolar epithelium, distention of alveoli by polymorphonuclear and mononuclear cells—observed in the psittacosis-infected lungs have been described in other infectious processes of this organ. Yet the peculiar combination of these pathological processes in lungs infected with psittacosis virus immediately distinguishes the reaction from ordinary pneumonias and from ordinary bacterial infections of the lungs.

From the evidence presented in the three preceding papers it is obvious (1) that parrots, regardless of the portal of entry of the inciting agent, rarely, if ever, develop a pneumonia as a result of an infection with the virus of psittacosis, (2) that rabbits and guinea pigs do not have an involvement of the lungs when inoculated intracerebrally, intradermally, or intraperitoneally, (3) that mice inoculated intracerebrally or intraperitoneally remain free from pulmonary lesions. Furthermore, in the present paper it has been shown that monkeys receiving the virus in the brain, although they become infected, develop no pneumonia. Opposed to such observations are those whereby it has been demonstrated that rabbits and monkeys, the former inoculated intratracheally, the latter infected either intratracheally or intranasally, exhibit pulmonary lesions. From these observations one is justified in surmising that psittacosis pneumonia in man probably results from the entry of the virus through the upper respiratory tract. Moreover, the results of the work reported in the first and second papers of this series indicate that the source of the virus in parrots and in man is the nasal and oral secretions and feces of the former and sputum of the latter. Thus in the case of man, a fair conception concerning the source of the infection and the portal of entry of the virus has been obtained. Consequently the precautions necessary for prevention of the infection are obvious. Despite them it is difficult, nevertheless, to prevent the spread of the disease from parrots to human beings, as witnessed by laboratory infections occurring under good conditions. Spread of the malady from man to man occurs infrequently, however. Certain possible reasons, such as amount of inoculum encountered or alteration in the virulence of the virus in the human host, may account for the apparent difference in the contagiousness of the disease.

CONCLUSIONS

The virus of psittacosis inoculated intratracheally or intranasally in monkeys produces a pneumonia similar to that caused by the same active agent in man.

Intracerebral inoculation of the virus induces a meningo-encephalitis characterized principally by a mononuclear reaction in the meninges.

Indirect evidence has been adduced to show that the portal of entry of the virus in man is the upper respiratory tract.

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EXPLANATION OF PLATES

Plate 12

Fig. 1. Monkey H, 2 days postinoculation. Roentgenogram of chest showing extensive mottling of right lower lobe with smaller shadows in the left lower. There is also evidence of spreading of the infection along the bronchial tree into the upper and middle lobes.

Fig. 2. Monkey K, roentgenogram of chest before inoculation.

Fig. 3. Monkey K, 5 days postinoculation. Two shadows at hilum on right side.

Fig. 4. Monkey J, 6 days postinoculation. Roentgenogram of chest: The right lung is almost completely involved, the left lower seems to be extensively consolidated, the left upper and middle lobes are relatively clear.

Fig. 5. Monkey I, roentgenogram before inoculation.

Figs. 6–10. Monkey I, roentgenograms showing the progression and regression of the consolidation. The pictures were taken 2, 5, 9, 11, and 13 days respectively after inoculation. Note rapid clearing within 48 hours, as evidenced by Figs. 9 and 10.

Fig. 11. Monkey D, roentgenogram before inoculation.

Fig. 12. Monkey D, 4 days postinoculation. Roentgenogram of chest showing involvement of a large part of the right lung and of the left lower lobe.

Plate 13

Fig. 13. Monkey H was sacrificed 2 days after inoculation. The painting shows a beginning pneumonia near the hilum. × 1.

Fig. 14. Monkey F died 6 days after inoculation. The painting reveals extensive pneumonia. Yet the edges of the involved lobes are not completely consolidated. Note the peculiar lilac-pink color. × 1.

Plate 14

Fig. 15. Monkey H, 2 days postinoculation. Section of lung showing spread of the infection along the alveolar walls. × 125. Eosin and methylene blue.

Fig. 16. Monkey H, 2 days postinoculation. Engorgement of blood vessels, serous exudate in alveoli, and polymorphonuclear leucocytes. × 450. Eosin and methylene blue.

FIG. 17. Monkey H, 2 days postinoculation. In this part of the consolidated lung, polymorphonuclear leucocytes are absent. Compare with Fig. 18. × 450. Eosin and methylene blue.

FIG. 18. Monkey H, 2 days postinoculation. Polymorphonuclear leucocytes are abundant in this portion of the involved lung. Compare with Fig. 17. × 450. Eosin and methylene blue.

FIG. 19. Monkey K, 7 days postinoculation (intranasal). Note the thick alveolar walls and the large mononuclear cells in the alveoli. Polymorphonuclear cells are absent. × 450. Eosin and methylene blue.

PLATE 15

FIG. 20. Monkey D, 4 days postinoculation. Section reveals different stages in the process of consolidation. The portion of the tissue least involved is near the periphery of the lobe. × 125. Eosin and methylene blue.

FIG. 22. Monkey D, 4 days postinoculation. Alveolar walls are necrotic. Fibrin and many polymorphonuclear cells are present. × 450. Eosin and methylene blue.

FIGS. 21, 23, 24. Monkey D, 4 days postinoculation. The alveolar walls are thickened and the alveoli contain desquamated epithelial cells together with other types of mononuclear elements. Very few polymorphonuclear leucocytes are seen. × 450. Eosin and methylene blue.

PLATE 16

FIG. 25. Monkey A, 8 days postinoculation. The alveolar walls are greatly thickened by cellular proliferation and infiltration. The cells lining the alveoli are swollen and some are seen free in the sacs. × 450. Eosin and methylene blue.

FIG. 26. Monkey A, 8 days postinoculation. The alveolar spaces contain various kinds of mononuclear cells. × 450. Eosin and methylene blue.

FIG. 27. Monkey A, 8 days postinoculation. It is difficult to distinguish the thickened alveolar walls from the cellular contents of the alveoli. × 450. Eosin and methylene blue.

PLATE 17

FIG. 28. Monkey I, 13 days postinoculation. At the time the monkey was sacrificed, resolution was progressing rapidly. Yet the section seems to indicate that organization had occurred. Compare with Figs. 29 and 30. × 450. Giemsa.

FIG. 29. Monkey I, 13 days postinoculation. Section showing resolving pneumonia. Many alveoli are greatly distended with air, while others still contain exudate. × 125. Aniline blue and orange G.

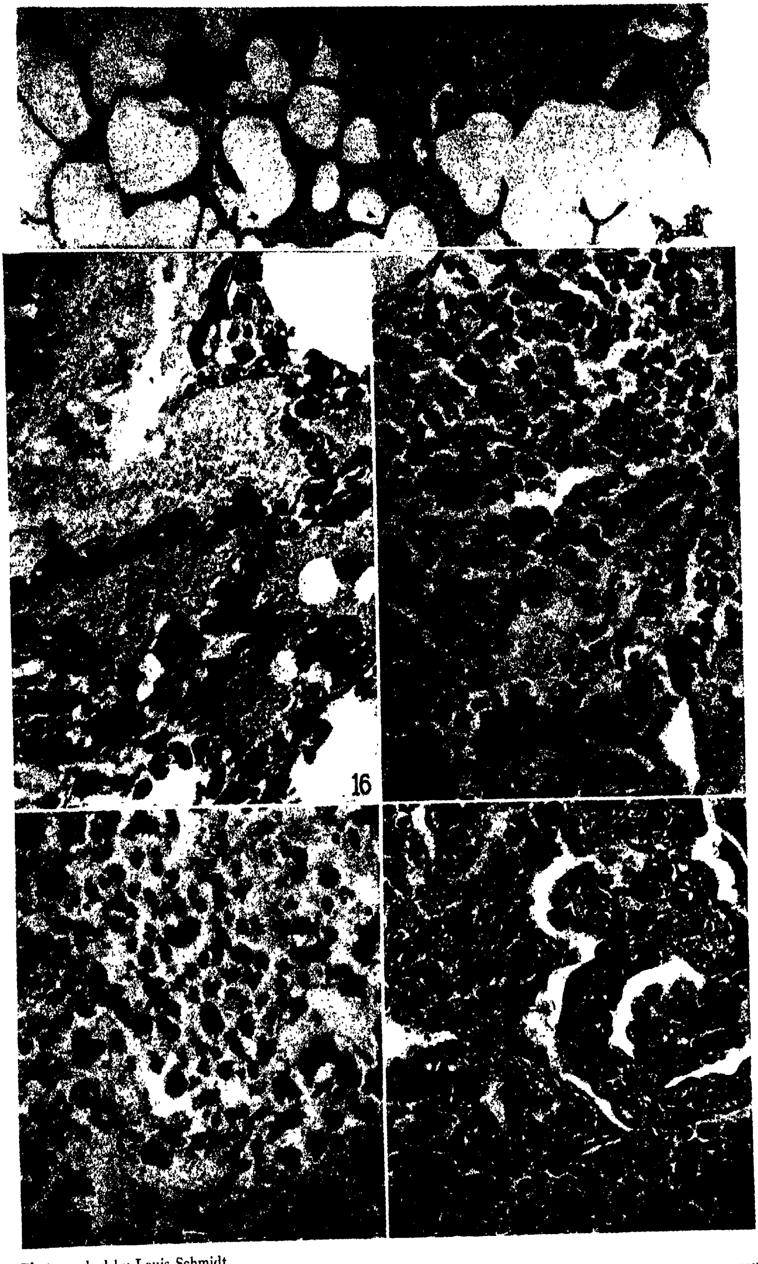
FIG. 30. Monkey I, 13 days postinoculation. The aniline blue and orange G stain clearly indicates that very little new connective tissue has been laid down. Compare with Figs. 28 and 29. × 450.

(Rivers and Berry: Psittacosis. IV)

13

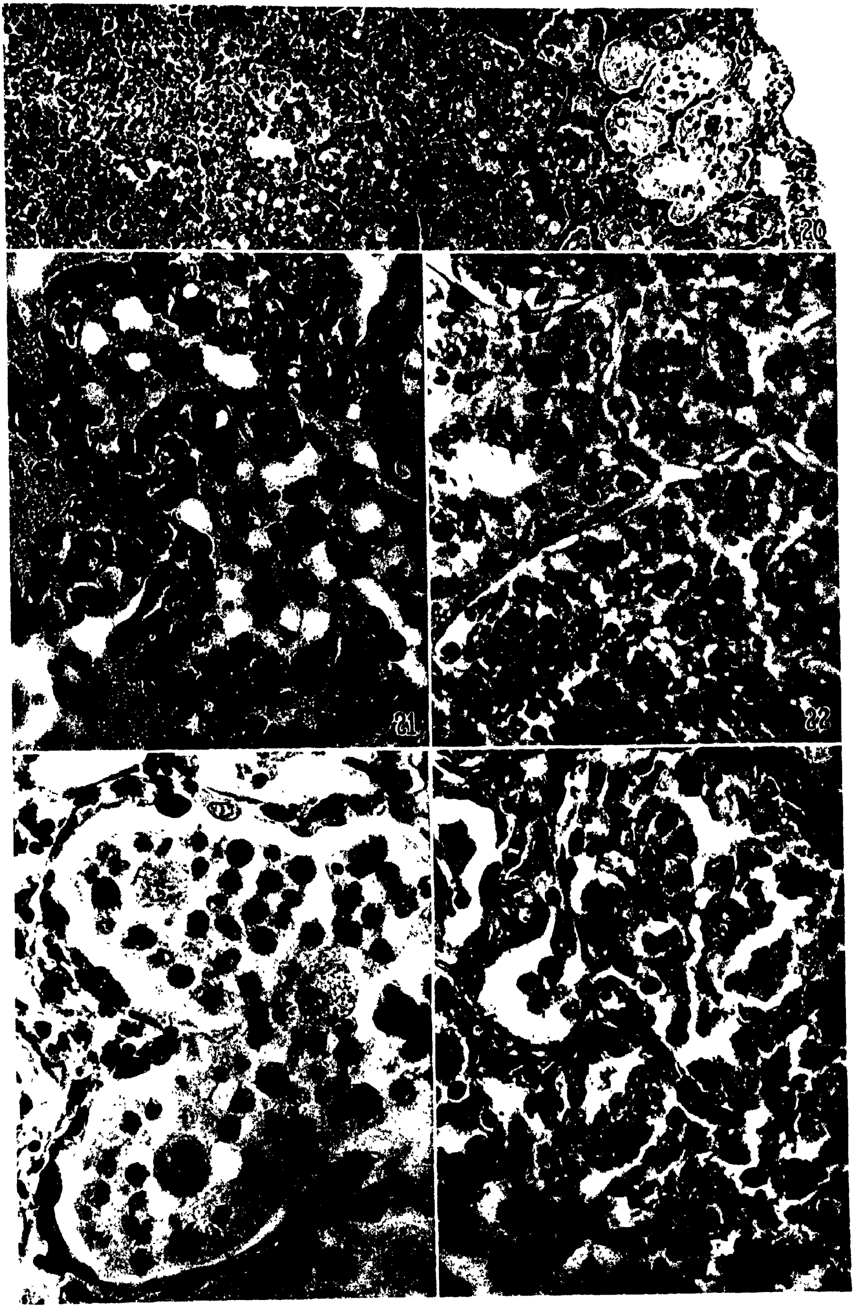
14

Painted by Louis Schmidt



Photographed by Louis Schmidt

(Rivers and Berry: Psittacosis. IV)



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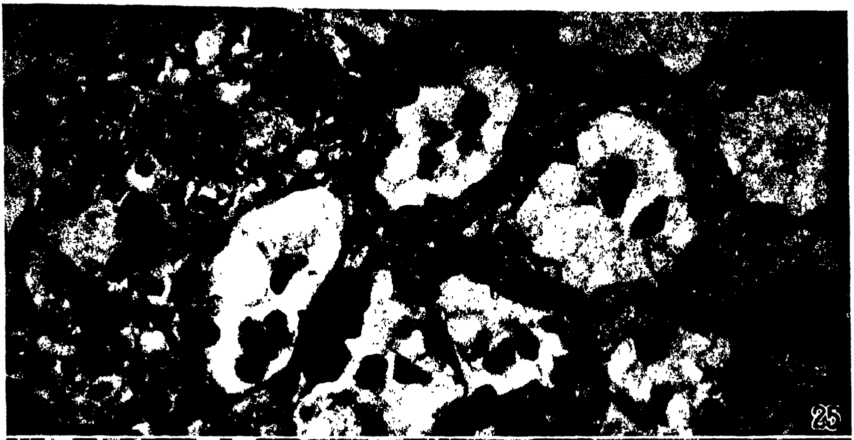

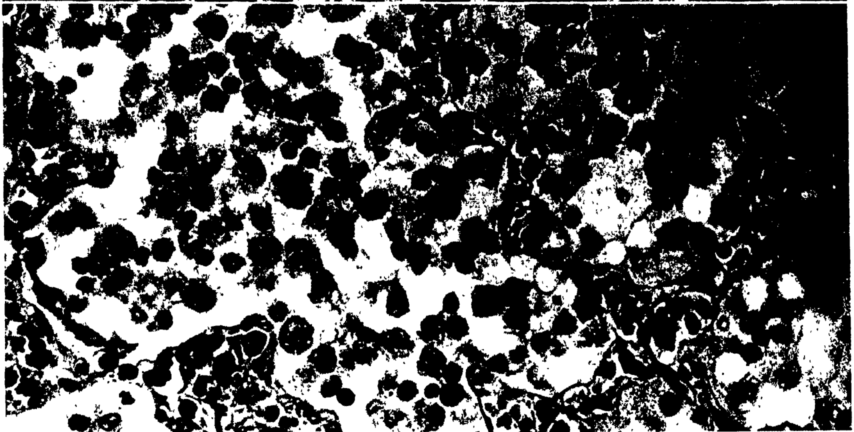

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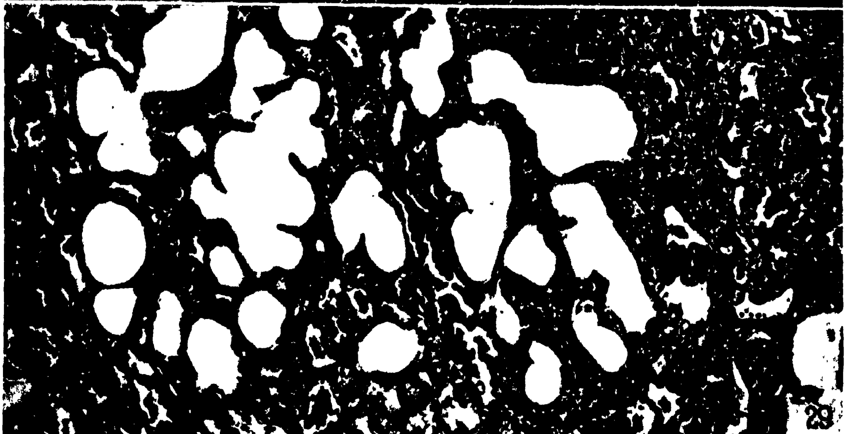
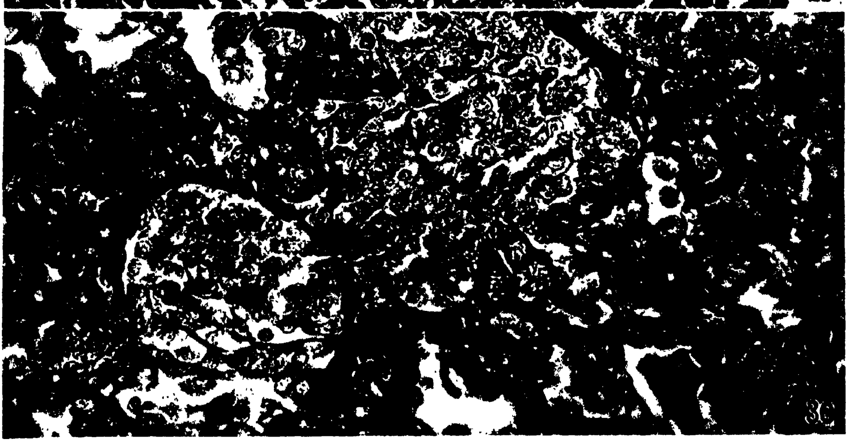
(Rivers and Berry: Psittacosis. IV)





Photographed by Louis Schmidt

(Rivers and Berry: Pstitacosis. IV)

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IDENTIFICATION OF REDUCING SUBSTANCES IN NEPHRITIC URINE

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In a study of the excretion of reducing substances in nephritis Hawkins, MacKay, and Van Slyke (1) found that the fermentable reducing substances were frequently increased in all types of the disease, and that in some of the degenerative cases the excretion amounted to a gross melituria.

The present work is an attempt to identify these fermentable reducing substances.

EXPERIMENTAL

Analytical Methods—The total and non-fermentable reducing substances in the urine were determined by the gasometric methods of Van Slyke and Hawkins (2) and recorded in terms of glucose.

Diurnal Excretion of Reducing Substances—In the two cases studied the daily amount of fermentable and non-fermentable reducing substances excreted was determined on several successive days. The diurnal excretion was determined on 3 hour specimens during a 24 hour period. The results are recorded in Figs. 1 and 2. The concentration of total reducing substances in the urine of Case I varied mostly between 0.84 and 0.96 per cent, with 0.12 to 0.15 per cent non-fermentable. In Case II the total reducing substances were about 0.20 per cent, of which 0.06 per cent was non-fermentable.

Decolorization and Removal of Protein from Urine—The urine samples used were slightly acid to litmus. Alcohol was added to make a 20 per cent alcoholic solution, since in such a solution the amount of reducing substances adsorbed on charcoal during decolorization is reduced from 20 per cent of the total to 3 per cent

(3, 4). To every liter of this solution about 10 gm. of norit were added; the mixture was shaken 15 minutes and filtered. The proteins were removed by adding to the filtrate twice its volume of 95 per cent alcohol and filtering. The filtrate was concentrated under diminished pressure.

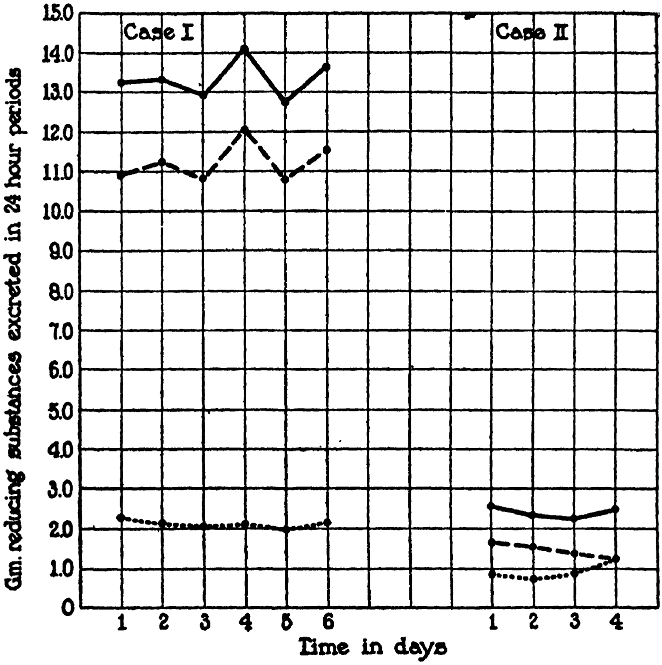

FIG. 1. Daily excretion of fermentable and non-fermentable reducing substances in two cases of nephritis. ———, total reducing substances excreted. — — —, fermentable reducing substance excreted. ...., non-fermentable reducing substances excreted.

An *osazone* was formed from the urine concentrated as described above by adding 2 parts of phenylhydrazine hydrochloride freshly recrystallized from 95 per cent alcohol and 3 parts of sodium acetate to 1 part of reducing substance calculated as glucose. The mixture was allowed to stand 15 minutes at room temperature in order to note any tendency to hydrazone formation, then placed in a boiling water bath for 2 hours. The osazones which had formed in the hot solution were filtered off immediately with suc-

tion, washed with hot water, then with 95 per cent alcohol, and with methyl alcohol. The crystals thus obtained were light yellow and were recrystallized once from 70 per cent alcohol. The melting point was estimated by raising the temperature about 1° per 10 seconds and correcting. The mixed melting point with glucosazone was also taken. The glucosazone was prepared as described above from pure glucose and recrystallized once from abso-

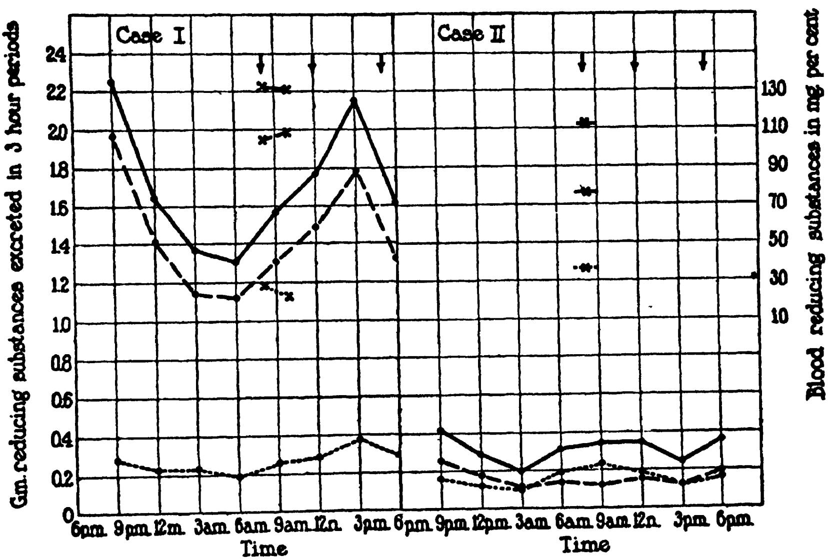

FIG. 2. 3 hourly excretion of fermentable and non-fermentable reducing substances over a period of 24 hours. .————., total reducing substances excreted. .— — — —., fermentable reducing substance excreted. ...., non-fermentable reducing substances excreted. ×————×, total reducing substances in blood. ×— — — —×, fermentable reducing substance in blood. ×........×, non-fermentable reducing substances in blood. Arrow designates time of meals.

lute alcohol. The mutarotation was determined by dissolving 0.0200 gm. of the osazone in 5 cc. of a mixture of 3 parts of dry pyridine to 2 parts of absolute alcohol. The results recorded in Table I show that the osazones from the urine differed from glucosazone only to the extent that might be expected from small amounts of impurities in the osazones from the urine.

Osazones Soluble in Hot Aqueous Solution. Urine 1—After removal of the large crystals of osazones which constituted the first

crop from the hot solution as described above, a crop of small, darker crystals was obtained on cooling. Similar crystals were obtained from the dark alcohol washings of the first osazone. The melting points of these osazones varied greatly and were indefinite (from 171–194°). The mutarotation in a mixture of pyridine and absolute alcohol was indicated by the following specific rotation.

Within 12 minutes....................................	$[\alpha]_D$ − 46.5
After 24 hours..	$[\alpha]_D$ − 23.2

Bromination—A portion of the urine prepared and concentrated as described above was treated with bromine for 48 hours with

TABLE I

Properties of Osazones Insoluble in Hot Aqueous Solution Obtained from Urine in Nephritis

Origin of osazone	Melting point (corrected)	Mixed melting point with glucosazone (corrected)	Mutarotation in pyridine-alcohol	
			Within 15 min. $[\alpha]_D$	After 24 hrs. $[\alpha]_D$
	°*C.*	°*C.*		
Glucose......................	207–208		−70.6	−20.3
Urine 1......................	204–205	203–204	−66.6	−20.5
" 2......................	202	202	−74.3	−19.8

occasional shaking. The amount of fermentable and non-fermentable reducing substances was estimated before and after treatment with bromine. The results are recorded in Table II. The fermentable reducing substance behaved like glucose in being completely destroyed by bromination. The non-fermentable reducing substances were unaffected by bromination. Everett and Sheppard (5) found that the reducing substance of normal urine is not destroyed by bromination.

Oxidation with Nitric Acid—A portion of the urine prepared as described above and containing about 3.5 gm. of reducing substances calculated as glucose was concentrated to about 10 cc. and treated with 2.5 times its volume of a solution containing equal parts by volume of nitric acid and water. After standing overnight the crystals of urea nitrate were filtered off with suction, the

filtrate was treated with more acid and evaporated on a large watch-glass on the water bath, with constant stirring and rubbing. As no mucic acid separated out, evaporation was continued to a paste, which was washed with a few cc. of water into a small beaker, neutralized with an excess of 50 per cent potassium hydroxide, then treated with an excess of glacial acetic acid. After standing in the ice box for several days the crystals were filtered cold, washed with 1 cc. of ice water, recrystallized from hot water, and dried in a vacuum desiccator at 70°. This procedure was essentially the same as that used by Heidelberger and Goebel[1] for the oxidation of aldobionic acid. The potassium acid salt was analyzed for potassium gravimetrically by conversion to potassium sulfate. The results check those calculated for the potassium acid salt of saccharic acid.

TABLE II

Effect of Bromination on Fermentable and Non-Fermentable Reducing Substances of Urine in Nephritis

Specimen	Treatment	Total reducing substances	Non-fermentable reducing substances	Fermentable reducing substances
		per cent	*per cent*	*per cent*
Urine 1	Before bromination	4.62	0.63	3.99
	After "	0.64	0.65	0
	Amount removed	3.98		3.99
Urine 2	Before bromination	0.43	0.21	0.22
	After "	0.18	0.18	0
	Amount removed	0.25		0.22

Calculated. K 15.75 per cent
Found. " 15.78 " "

Precipitation of Reducing Substances with Copper Sulfate and Calcium Hydroxide—An attempt was made to separate the reducing substances with copper, in the hope of separating glucose from the other constituents. The method developed by Van Slyke (6) for precipitating glucose with copper sulfate and lime was used. For every 125 cc. of Urine 1 concentrated as described above and containing about 1 per cent of reducing substances calculated as

[1] Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, 70, 613 (1926).

glucose, 50 cc. of a 20 per cent solution of copper sulfate were added, followed by a 10 per cent suspension of calcium hydroxide, which was added with shaking until the reaction was alkaline to litmus. After standing 30 minutes the precipitate was filtered off with suction, suspended in water, and acidified with sulfuric acid. The copper was removed with hydrogen sulfide and the filtrate concentrated under diminished pressure. Both total and non-fermentable reducing substances were estimated before and after hydrolysis with 0.2 N sulfuric acid for 1 hour, and also before and after bromination. Only 42 per cent of the reducing substances was recovered from the precipitate, but non-fermentable as well as

TABLE III

Reducing Substances Precipitated from Urine 1 with Copper Sulfate and Calcium Hydroxide

Treatment	Total		Non-fer-ment-able	Fer-ment-able	Increase or decrease, ferment-able	Increase or decrease, non-fer-mentable
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before precipitation	7.46					
After " *	3.13	0.735	0.112	0.623		
" " + hydrolysis*		0.789	0.140	0.649	+0.026	+0.028
" " + bromination*		0.165	0.153	0.012	−0.611	+0.041

* Figures represent material recovered from the copper-lime precipitate, not from its filtrate.

fermentable substances were recovered in practically the same proportions that were originally present in the urine. These results are shown by comparison of Table III with Fig. 1. Hydrolysis of the precipitated fraction showed a slight increase in reducing substances, both for the fermentable and the non-fermentable fraction. Bromination caused a loss of 98 per cent of the fermentable reducing substance, and a slight increase in the non-fermentable fraction; the latter change was probably caused by hydrolysis with hydrobromic acid formed by the bromine in aqueous solution.

Non-Fermentable Reducing Substances. Urine 1—After treatment with norit, removal of proteins, and concentration, the urine was treated with yeast until all fermentable reducing substance

was removed. The time required was ½ hour. The fermented solution was subjected to a series of tests for various carbohydrate groups and to hydrolysis. The results are recorded in Table IV.

Osazones were formed as described above both before and after hydrolysis. The osazones in this case appeared only slowly in the cooled solution. No osazones could be obtained from yeast sim-

TABLE IV

Non-Fermentable Reducing Substances of Urine 1

Reactions		Time of hydrolysis	Reducing substances
		hrs.	*per cent*
Molisch test for carbohydrates	+		
Phloroglucinol test for pentose	−		
Resorcinol test for ketose	−		
Naphthoresorcinol test for glucuronic acid	+		
Hydrolysis with 0.5 N HCl		0	1.28
		3	1.34
		6	1.24
		12	1.20

TABLE V

Properties of Osazone Obtained from Urine 1 after Fermentation with Yeast

Treatment	Melting point (corrected)	Mutarotation	
		Within 20 min. $[\alpha]_D$	After 48 hrs. $[\alpha]_D$
	°C.		
Before hydrolysis	186–194	−60.6	−36.4
After 30 min. hydrolysis in 0.2 N H_2SO_4	194–203	−35.0	−28.0

ilarly treated. The properties of the osazones are recorded in Table V.

The osazones (Table V) were soluble in acetone and had variable and indefinite melting points. Both of these properties are characteristic of the product formed by the reaction of phenylhydrazine on glucuronic acid, which forms a series of compounds (hydrazone, hydrazide, hydrazone hydrazide, osazone hydrazide) with melting points between 107–217° (7–11).

A further separation of the non-fermentable reducing fraction was attempted through the precipitation of glucuronates with basic lead acetate. The fermented Urine 2 concentrated as described above, was treated first with neutral lead acetate to precipitate phosphates, sulfate, and other impurities which would otherwise be included in the glucuronate precipitate. The glucuronates were then precipitated with basic lead acetate. The

TABLE VI

Effect of Hydrolysis on Reducing Substances Precipitated by Basic Lead Acetate

Treatment	Time of hydrolysis	Reducing substances
	min.	*per cent*
Before hydrolysis	0	0.57
After "	10	0.69
" "	30	0.64
" "	60	0.66

TABLE VII

Properties of Osazone Obtained from Urine 2 after Fermentation with Yeast and Treatment with Basic Lead Acetate

Age of osazone	Melting point (corrected)	Mixed melting point with glucosazone (corrected)	Mutarotation in pyridine-alcohol	
			Within 15 min. $[\alpha]_D$	After 24 hrs. $[\alpha]_D$
	°C.	*°C.*		
Freshly prepared	204–205			
After 1 wk	184–194	184–194	−48.8	−14.4

precipitate, after suspension in water and removal of lead with hydrogen sulfide, reduced Benedict's solution and gave a strong naphthoresorcinol reaction. The precipitation process was repeated. The solution was evaporated to dryness under diminished pressure, taken up in methyl alcohol; insoluble substances were removed by centrifugation, and the alcohol by evaporation under diminished pressure. The complete removal of acetic acid by distillation under diminished pressure was unsuccessful, as the solution became colored and the reducing power decreased.

An aqueous solution of the reducing substances was hydrolyzed with 0.2 N sulfuric acid and analyzed at intervals for 1 hour. The results are recorded in Table VI.

The formation of a combination with parabromophenylhydrazine was unsuccessful, probably because of insufficient material. A brown sticky mass was obtained instead of crystals. An attempt to form a cinchonine combination was likewise unsuccessful.

The filtrate from the first basic lead acetate precipitation was acidified, treated with hydrogen sulfide to remove the lead, neutralized, and treated with phenylhydrazine. The osazone appeared slowly on cooling, and was recrystallized from 30 per cent alcohol. The osazone decomposed rapidly on standing in a desiccator, so that by the time the mixed melting point was taken the crystals were dark brown and the melting point very indefinite. The melting points and mutarotation are recorded in Table VII.

DISCUSSION

Among the sugars which form osazones with melting points between 200–208° (Table I) are glucose, levulose, mannose, lactose, and maltose. The levorotation of the osazone ruled out maltose, the osazone of which is dextrorotatory. Lactose was ruled out by the fact that no mucic acid was formed on oxidation with nitric acid. Glucose, levulose, and mannose form identical osazones, which therefore have the same melting point, the same mutarotation, and which, when mixed with glucosazone, show no change in melting point. Since the osazones from both urines showed mutarotation in a mixture of pyridine and absolute alcohol similar to that of glucosazone (Table I), and the mixed melting point with glucosazone showed no change from the original melting point, it can be concluded that the sugar is identical with one of these three. Mannose, however, forms a hydrazone on standing at room temperature for 15 minutes with phenylhydrazine. Such a reaction could not be demonstrated with either of the urine samples. The differentiation between glucose and levulose was made by means of bromination. Bromine will oxidize 98 per cent of the aldo groups present in an aqueous solution, but has no effect on the keto groups. Table II shows that all fermentable reducing substance was completely removed from the concentrated urine in both cases; therefore, the fermentable fraction is entirely composed of sugar con-

taining aldo groups. The non-fermentable fraction was unaffected by bromine. Since this reaction ruled out levulose, it follows that the similarity of these osazones with glucosazone in melting point, mixed melting point, and mutarotation points to the identification of the fermentable sugar in these two urine samples as glucose. This identification was further substantiated by the oxidation of the sugar with nitric acid and the formation of a potassium acid salt of the oxidation product, which proved on analysis to be the potassium acid salt of saccharic acid.

The more soluble osazones obtained by slow cooling as a second crop were small in amount and dark in color. The melting points were indefinite and varied greatly, from 171–194°. The mutarotation was shown by the change from −46.5 or $[\alpha]_D$ in pyridine-absolute alcohol within 12 minutes to −23.2 after 24 hours. From the mutarotation an impure glucosazone or a mixture of osazones might be suspected. Since glucosazone is not entirely insoluble in hot water or cold alcohol, it is likely that some glucosazone was present in this fraction.

SUMMARY

The fermentable reducing substance excreted in the urine of two patients with nephritis is identified as glucose.

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THE ETIOLOGY OF INFECTIOUS DIARRHEA (WINTER SCOURS) IN CATTLE

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To judge from information from various sources there exists in certain dairying sections of this country an epidemic form of diarrhea among cows. Such outbreaks are said to occur during the fall and winter months and for this reason the disease is frequently referred to as winter dysentery. Little is known concerning its ætiology, and although both Steffen (1) and Marshall (2) recognize it as an infectious disorder, others hold the view that it is a dietary disease without specific cause. In the main the disease is said to be an afebrile one characterized by the passage of dark brown or blackish brown liquid feces, and because of this fact it is known in certain localities as black scours. The mortality varies; Steffen states that the condition is rarely fatal but Marshall reports 16 deaths among 62 affected cows. All agree that the disease seriously affects the milk yield both during and subsequent to the attack.

The Characterization of the Disease

In all we have had access to 5 herds in which a total of 400 cows were affected.[1] In the main there was relatively little fever although in one small group in which three fatalities occurred two had temperatures of 39.6° and 40.2°C. respectively. The onset appears suddenly; usually a few cows begin to scour, then the diarrhea may spread rapidly throughout the particular group or, as we observed in two

[1] We wish to acknowledge our indebtedness to Drs. J. H. Herron of Bordentown, N. J., E. H. Hopper of Ridgewood, N. J., and G. H. Kimnach of Hightstown, N. J., through whose cooperation we were able to visit herds in which the disease existed.

outbreaks, to all the adults in the herd. As a rule the calves are not appreciably affected.

In general the more severely affected animals are dull, refuse food, and at times exhibit signs of abdominal pain. The feces are fluid, in some cases fetid, usually deep brown to greenish black in color, and often contain blood and mucus. The milk yield is greatly diminished. The diarrhea usually runs a relatively short course from a few hours to 4 or 5 days, when the feces become firmer, the general health improves, and the milk yield begins to increase. In certain instances cows apparently recover but when placed on full diet diarrhea recurs. At times the onset is accompanied by a dry cough and excessive salivation. As a rule the respirations are rapid.

Four cases were autopsied. One was from a small herd where two other cows had died of the disease, two others were slaughtered when acutely ill, and the fourth was a cow which had suffered from an acute attack and after apparent recovery again scoured when heavily fed.

Examination of material from these individuals indicated a catarrhal inflammation of the small intestine (jejunum and ileum) inasmuch as the serosal vessels were injected, the intestinal walls edematous, and the mucosa swollen, wrinkled, and congested. The intestinal content was fluid, usually dark brown in color, and admixed with mucus. The liver in three instances was ochre color, dry in consistency, and readily fractured when bent, and the gall bladder was engorged with dark bile. The heart muscle was dry and brownish red in color. In addition the folds of the abomasum were congested and edematous. The mesenteric lymph glands were enlarged, pale, and juicy.

Histologically well marked changes were observed in the sections of the small intestine where a well defined vascular engorgement was noted. The superficial mucosa was degenerated and overlain with mucus and disintegrated cells. The mucosa was infiltrated with round cells and leucocytes, the vessels of the submucosa engorged, and the connective tissue edematous. Sections of the liver in three of the four cases revealed hydropic degeneration of the parenchymatous cells.

Bacteriological Findings from the Spontaneous Cases

Cultures from liquid feces from acute cases in all outbreaks were made on lactose agar plates containing indicator. From the cows in three herds actively motile Gram-negative rods which failed to ferment lactose were obtained, and at times such organisms made up 90 per cent of the organisms on the plates. From other cases in the same herd they could not be cultivated. Later organisms of this type were correlated with *B. coli mutable* and when fed to healthy calves failed to establish themselves in the intestinal tract.

Blood cultures from acute cases failed to develop suggestive organisms. Media inoculated with bits of organs from the slaughtered animals remained sterile, and inoculations from the intestinal content and mucosa of various portions of the small intestine revealed either *B. coli* or mutable colon bacilli in enormous numbers from all portions of the small intestine.

The only evidence indicating that the disease was an infectious one seemed to lie in the fact that it spread from group to group, but this fact might be explained by some toxic substance in the food or drink. It seemed essential to attempt to reproduce the disease, and our first protocols deal with such experiments.

EXPERIMENTAL

A 4 months old calf (1629) was fed with a small quantity of intestinal content obtained from the jejunum and ileum of a spontaneous infection. The spontaneous case originated in a large dairy herd where diarrhea had been prevalent for 6 weeks. This case was one of the last to appear and after apparent recovery again had diarrhea when heavily fed. The calf developed diarrhea 3 days after feeding, the attack lasting 2 days. Diarrhea was noted again on the 7th and 14th days. The animal was slaughtered 16 days after the feces were administered.

At autopsy a well defined inflammation involving the duodenum, jejunum, and upper portion of the ileum was found. The lesions consisted in a well marked engorgement of the vessels of the serosae. The intestinal walls were thickened. The mucosa was swollen and varied from bright red to pink in color. The content was slimy and tinged with bile. Peyer's patches and solitary follicles were frequently swollen and often overlain with deep red swollen mucosa. There was little abnormal observed in the large intestine. The spleen, liver, and kidneys appeared normal.

Histological examination of fixed and stained material revealed well defined changes in the small intestine; frequently the superficial mucosa was degenerated and often overlain with mucus, leucocytes, and necrotic cells. The capillaries of the mucosa were engorged and leucocytes had invaded the mucosa in large numbers. The secreting layer of the mucous glands had been invaded by leucocytes and aggregates of such cells at times plugged the lumen of the gland. The submucosa was congested and edematous.

The inoculation of lactose agar plates with the contents of various segments of the small intestine revealed large numbers of *B. coli* throughout the jejunum and ileum but no other significant organism.

Bits of the mucosa from the jejunum were washed in several changes of sterile salt solution, ground in a glass grinder similar to that recommended by Hagan (3), and the ground material mixed with sterile salt solution. This suspension was

inoculated into the condensation fluid of tubes of slanted agar to which a few drops of horse blood agar had been added. From the first tube two others in series were inoculated. All tubes were then sealed with sealing wax and after suitable incubation the condensation fluid was examined. Frequently the tubes inoculated directly with the suspension developed rapidly growing organisms, but from the secondary tubes what appeared to be pure cultures of tiny motile vibrios were obtained.

The next calf (1655) was fed liquid feces, mixed with salt solution, from three acute cases of diarrhea. The spontaneous disease had attacked about half the herd up to the time of our visit and within the next week practically every animal developed the disease. The feces which we fed to Calf 1655 contained besides *B. coli*, *B. coli mutable* in considerable numbers. Little of note indicative of enteritis was observed as the result of the artificial infection except that the calf was constipated, the feces clay colored and always contained large masses of clay colored mucus. The animal was slaughtered 12 days after the feces were fed. On autopsy the liver was yellowish brown in color and friable, and on section the color extended throughout. The cut surfaces were granular. The upper and middle portions of the jejunum revealed gross changes similar to those observed in the intestines of Calf 1629. In addition the mucosa of the ileum was swollen and reddened for a distance of 1 m. above the ileocecal valve. Edema and congestion of the leaves of the abomasum were also noted.

Essentially the same histological picture as that encountered in the case of Calf 1629 was noted in the fixed and stained material from Calf 1655. In the latter case the liver revealed distinct changes such as nuclear degeneration of the liver cells and hydropic infiltration of the cytoplasm of such cells, and passive congestion.

Nothing significant developed in the lactose agar plate cultures inoculated with content of the jejunum and ileum. *B. coli* was not found above the middle ileum.

Media inoculated with bits of liver, spleen, and kidney remained sterile. Fragments of the inflamed mucosa of the jejunum were treated in the manner previously described, and when ground, suspended in broth, and inoculated into blood agar, developed after suitable incubation under seal vibrios that resembled those obtained from Calf 1629.

The third calf (1641) when 16 days old was fed feces mixed with salt solution from three acute cases of diarrhea. This material was obtained from another large herd where the disease within the course of 7 days attacked over 160 animals. The scouring was severe in the milch cows but relatively mild in the bulls and young stock. The feces contained no cultivable organism of the colon group other than *B. coli*. Within 24 hours the calf was depressed, and on the 2nd day there was an elevation of temperature (39.5°C.). On the 3rd day the depression was more marked, and the feces were soft, yellow in color, and fetid. The calf's condition improved on the 4th day and it was slaughtered on the 5th day. Autopsy revealed an orange-red liver with rounded borders. There was a well marked patchy inflammation of the mucosa of the small intestine extending

throughout the jejunum and into the anterior half of the ileum. Here the mucosa was reddened, swollen, and frequently overlain with tenacious mucus. The folds of the abomasum were swollen and reddened. The intestinal content was yellowish white in color and contained large quantities of mucus.

Histological examination of sections of the liver revealed well marked cloudy swelling of the parenchymatous cells. The histological changes encountered in sections of the intestine were similar to those found in the two other calves.

B. coli as indicated by lactose agar plate cultures was present in large numbers throughout the ileum and in smaller numbers in the jejunum. Pure cultures of actively motile vibrios developed in the tubes inoculated with the washed mucosa of the upper jejunum but from the middle jejunum and ileum only mixed cultures were obtained.

The experiments indicated that when feces or intestinal content from cases of diarrhea were mixed with salt solution and administered to calves by mouth a definite reaction followed. In two instances a slight temperature reaction accompanied by general depression occurred but in all cases a catarrhal enteritis was found at autopsy. In two instances the feces became soft and fetid and in the other case the feces contained large quantities of mucopurulent material. The administration to calves of feces from spontaneous cases resulted then in a disease of the small intestine accompanied by a mild general reaction. The disease although less severe simulated the malady encountered in the older cows.

In addition from the experimental cases we had succeeded in obtaining cultures of tiny, actively motile vibrios from involved portions of the intestine which might be of etiological significance. It is of interest to note that we had seen organisms of similar morphology in preparations of mucus obtained from the feces of cows with diarrhea and that they had been found in small numbers in mixed cultures made from bits of this mucus.

To establish the relationship of these vibrios to the disease a series of experiments were undertaken. The results are recorded in Table I.

It will be noted that the cultures when mixed with the feed or suspended in salt solution and administered by mouth were capable of producing in most instances recognizable symptoms. Inflammation of the mucosa of the jejunum and anterior ileum was a constant finding. From two of the experimental calves (1669 and 1686) a large number of pure cultures of vibrios were cultivated. Cultures from

TABLE I

The Effect of the Aural Administration of Vibrios

Animal	Age	Fed	Symptoms	Autopsy	Bacteriological findings
Calf 1669	26 days	2 cultures Calf 1629 1 blood agar Calf 1641 1 leptospira Calf 1655	Rise of temperature, diarrhea with mucus for 3 days. Killed on 7th day	Cloudy swelling of liver cells; well marked inflammation of lower duodenum, jejunum, and upper half of ileum	Many cultures of vibrios from two portions of jejunum
Cow 1683	15 mos.	5, 72 hr. blood agar cultures of vibrios from Calf 1669, 3rd transfer	Rise of temperature, suppression of milk. Passage of bloody mucus containing vibrios, mild diarrhea on 5th day. Killed on 6th day	Inflammation of the mucosa of jejunum and ileum	Vibrios mixed with spore-bearing bacilli. Pure cultures not obtained
Calf 1686	15 days	2, 48 hr. blood agar cultures from Calf 1669, 13th transfer	Little general reaction, large quantities of mucopurulent material in feces. Killed on 5th day	Severe inflammation of jejunum and upper half of ileum	Vibrios in large number throughout jejunum and upper ileum
Calf 1630	3½ mos.	3 blood agar cultures from Calf 1655	Diarrhea during first 3 days, rapid recovery. Killed on 8th day	Mild inflammation of jejunum and upper ileum	Vibrios not obtained

the young cow (1683) also contained vibrios but in every instance mixed with rapidly growing bacilli from which they could not be freed. The other calf (1630) developed a mild diarrhea and at autopsy the lesions although not pronounced could be recognized but the organism could not be cultivated.

The only other organisms which might be regarded as of etiologic significance were the mutable colon bacilli. In certain cases they comprised over 90 per cent of the organisms present in the plate cultures made from feces or intestinal contents but in many cases they were not found. Young broth cultures in amounts of 35 and 40 cc. were administered to calves but as far as we could determine they failed to establish themselves in the intestinal tract. In addition the feces fed to Calf 1641 contained mutable colon bacilli in large numbers but we failed to find them in the fecal cultures or in the intestinal tract at autopsy.

DISCUSSION

Our studies indicate that there exists an infectious disease of cows characterized by severe diarrhea accompanied by marked suppression of milk secretion. In general the same type of symptoms were noted among animals in widely separated herds, and when it was possible to obtain material at autopsy the same lesions were found. Mention has been made of the observations of Steffen and Marshall who characterize the disease with which they dealt as an epizootic dysentery. Our experience indicates, to judge from the natural and experimentally produced disease, that the inflammation has a different distribution from that encountered in human dysentery. The small intestine, particularly the jejunum and upper ileum, seem to be the principal locus.

That the condition is an infectious one there can be little doubt. However, for the purpose of discussion, attention must be given to dietary factors. As a rule the first portion of the ration to be looked at askance is the ensilage. In the first outbreak we encountered the sick received the same ensilage as the older cows in which there was no diarrhea. In another no ensilage had been fed, the animals receiving only the food to which they were accustomed. Two other outbreaks afford strong arguments that the disease is infectious. It is true that

in one of these some of the first cases occurred among cows fed from an old lot of ensilage; nevertheless cows in the same barn fed beet pulp instead of ensilage came down with the disease, and 2 or 3 days later diarrhea appeared among the cows in other barns where there was no question raised as to the quality of the feed. Cows on outlying farms were later affected, where all feed stuffs were of different lots. Evidence in another instance is indicative of the infectious nature of the malady. Here the first cases occurred among the cows in a wing of the main barn, the disease next appearing in the main barn and other large barns connected by runways with it. There had been no change in the character of the feed prior to the outbreak. Direct proof of the infectious nature of the diarrhea is afforded by the findings here recorded when calves were fed suspensions of feces from spontaneous cases. Two of the calves developed diarrhea and the third clinically manifested enteritis by the passage of large quantities of mucus covered feces and mucopurulent material. All three when killed revealed well defined characteristic inflammation of the small intestine. In all three cases vibrios similar in morphology were obtained from the intestinal tract.

In regard to the nature of the etiological agent it may safely be said that the more common types of organisms which would be suspected in such a disease have been ruled out by the methods employed. If organisms of the colon-typhoid group were responsible it is probable that our methods would have demonstrated them, since we readily cultivated *B. coli mutable* from the feces or intestinal tract of certain spontaneous cases. These when fed to calves in amounts as high as 45 cc. of 18 hour broth cultures failed to produce disturbances or, as far as we could tell, to establish themselves in the intestinal tract.

The only organisms encountered which appeared to offer etiologic possibilities were the vibrios isolated from the inflamed intestines of calves which developed enteritis as the result of the ingestion of feces from spontaneous cases. Such organisms had been seen in preparations of mucus obtained from spontaneous cases and in one instance vibrios had been found in mixed cultures made from mucus obtained from the feces. Three calves when fed these cultures developed diarrhea or other evidences of enteritis. A young cow fed a single strain recently isolated from one of the experimental calves developed diar-

rhea with blood stained mucus in the stools, accompanied by almost complete suppression of milk secretion. All four cases had on autopsy an enteritis similar to that encountered in the spontaneous disease and in the calves fed feces from spontaneous cases. From the intestines of two calves fed these cultures vibrios were recovered in pure culture. From the cow vibrios were obtained in mixed culture only, and from the third calf the tubes inoculated with suspension of inflamed intestinal mucosa remained sterile.

SUMMARY

A disease of cows manifested by severe diarrhea has been described. The condition is characterized by the frequent passage of dark brown or black feces, often containing mucus and blood. The principal lesions are catarrhal inflammation of the small intestine and liver degeneration.

By feeding feces from spontaneous cases to calves a similar but milder disease characterized by the same type of enteritis was produced. Vibrios were cultivated from the inflamed intestinal tract of such experimentally induced cases. Pure cultures of the vibrios when fed to other calves, in certain instances, produced diarrhea and a well marked enteritis similar to that observed in both the spontaneous disease and in calves following the feeding of feces from naturally affected cows. Vibrios were recovered from the inflamed small intestine of three out of four animals fed such cultures.

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VIBRIONIC ENTERITIS IN CALVES

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In a previous paper (1) we called attention to a disease of adult cows characterized by profuse diarrhea from which by indirect means we obtained vibrios and these when fed to healthy calves produced a well defined enteritis. It seemed likely that organisms of this class might be responsible for certain forms of enteritis in calves, especially inasmuch as various types of intestinal disease of obscure etiology are known to exist. That vibrios can be the cause of a more or less chronic intestinal disease is brought out in the present paper.

The Disease as Encountered among Calves

During the latter months of 1929 and 1930 many of the older calves in a large dairy herd were affected with a complication of diseases. Through the kindness of Dr. Theobald Smith who studied the respiratory aspect of the problem, we obtained several gastrointestinal tracts of calves suffering with pneumonia but later many cases were encountered where the cause of death or general debility was directly correlated with intestinal disease.

As a rule calves over 2 weeks old were affected. The unthrifty appearance of such animals attracted attention. The tail and buttocks were often covered with feces and the abdomen distended. The feces were soft or gummy in consistency and varied from clay colored to dark brown in color. They frequently contained blood and mucus and when suspended in water were noted to contain irregular, firm, sticky masses, and these when crushed were found on microscopic examination to contain large numbers of leucocytes often embedded in mucus. As a rule the disease as we have encountered it is more or less chronic but a few cases dying after a short illness have been seen. During the later portion of 1929 and the early months of 1930 only a few sporadic cases appeared but with the onset of colder weather a well marked outbreak occurred, and the disease has been

largely responsible for the slaughter or death of many calves. We have tabulated the more important pathological and bacteriological findings of a number of cases.

TABLE I

The Distribution of Intestinal Lesions and Bacteriological Findings in the Spontaneous Disease

No. of calf	Age	Distribution of lesions	Bacteriological findings	
			Vibrios	The distribution of *B coli* as determined by lactose agar plates
	days			
1652	18	Inflammation of small intestine	Pure culture from lower jejunum and upper ileum	
1682	57	Patchy inflammation of jujunum and upper ileum	Mixed culture of vibrios from jejunum	*B. coli* throughout jejunum and ileum
1699	50	Inflammation most marked in middle jejunum and upper ileum	Vibrios in pure culture from middle jejunum and upper ileum	*B. coli* and streptococci from middle jejunum and ileum
·1700	28	Lower half of jejunum and upper half of ileum	Vibrios in pure culture from the middle jejunum	*B. coli* not found in jejunum
1728	18	Inflammation throughout jejunum. Severe inflammation in ileum extending close to ileocecal valve	Pure culture from middle jejunum. Vibrios mixed with *B. coli* in ileum	Middle jejunum sterile. *B. coli* present in middle ileum
1731	68	Inflammation throughout small intestine	Vibrios not obtained from intestine. All tubes inoculated with bits of liver developed vibrios	*B. coli* throughout ileum
1745	51	Patchy inflammation of jejunum. Severe inflammation throughout ileum	Vibrios in pure culture from lower ileum	*B. coli* as far as the middle ileum
1748	56	Inflammation of the abomasum and small intestine	Pure cultures of vibrios from duodenum and mixed from stomach and remainder of small intestine	*B. coli* in liver and small intestine below the middle jejunum

From Table I it can be said that most of our material was obtained from calves between 18 and 68 days old, but in all probability the ani-

mals are infected relatively early in life. Mention has been made of the unthrifty appearance of the calves frequently accompanied by chronic diarrhea. Autopsy reveals well marked intestinal lesions.

The folds of the gastric mucosa (abomasum) are often reddened and swollen. The small intestine, from the duodenum to the ileum, reveals well marked changes. The vessels of the serosae are injected and the intestinal walls appreciably thickened. The mucosa is reddened, the folds enlarged, and the solitary follicles swollen and overlain with reddened mucosa. The content is rich in mucus, desquamated epithelial cells, and leucocytes. The liver when involved is yellowish brown in color and fractures easily. The gall bladder is usually well filled with normal appearing bile. The mesenteric lymph glands are pale and enlarged. Vibrios are at times readily demonstrated in properly prepared films from involved portions of the intestine. In other cases they are not readily found. Histologically the intestinal lesions consist of superficial degeneration of the mucosa, engorgement of the vessels of the mucosa, and infiltration of the cores of the villi and mucous layer with round cells and leucocytes. Leucocytic infiltrations into the lumen of the mucous glands are not uncommon, and edema of the lymphoid structure of the solitary follicles with leucocytic infiltration, congestion, and edema of the submucosa is usually found. At times hyperplasia of the endothelial elements of the submucosa has been encountered. The liver cells may be degenerated and infiltrated with fat.

Since most of our cases were regarded as of some duration, it seemed desirable to endeavor to ascertain the nature of the acute disease as it occurred spontaneously. With this in view young vigorous calves were mingled with those presenting chronic symptoms and later slaughtered at an abattoir. The more important details are given in Experiment 1.

Experiment 1.—Three calves ranging in age from 6 to 8 days were housed with a 2 months old calf which presented symptoms of chronic enteritis. Vibrios had been obtained from the inflamed intestines of older calves kept in the same pen. Two younger calves from the same source as those exposed to natural infection were slaughtered at the time the other three were exposed. Their intestinal tracts appeared normal and vibrios were not found. The calves were under observation for periods of 14, 16, and 21 days respectively.

Calf 1707 had fever and diarrhea 10 days after exposure and the feces contained blood. Diarrhea persisted until the 14th day when the calf was slaughtered. Characteristic severe inflammation of the upper and middle jejunum and the lower portion of the ileum was found. Many apparently pure cultures of vibrios were obtained from the washed and ground mucosa from inflamed regions. In addition pure cultures of vibrios were also obtained from the liver. *B. coli* was

present in the content in large numbers throughout the ileum and in the lower jejunum; above this region it was present in smaller numbers.

10 days after exposure the feces of Calf 1709 were soft and contained blood. The calf had fever. It was slaughtered 21 days after the experiment started. A moderately severe enteritis which began in the upper portion of the jejunum and extended practically throughout this region of the bowel was encountered. A more severe inflammation was observed in the lower portion of the ileum. Vibrios were obtained from the upper and middle jejunum but below this point the cultures were overgrown with the ordinary intestinal forms. Cultures from the liver failed to show vibrios but all contained anaerobes. Lactose plate cultures revealed *B. coli* in large numbers throughout the jejunum and ileum.

Calf 1710 revealed symptoms of gastric disorder and scoured on one occasion. When slaughtered on the 14th day it revealed a mild patchy congestion of the mucosa of the upper jejunum and ileum. Vibrios were not found in the cultures from the intestinal tract and liver. *B. coli* was present in the content in large numbers throughout the small intestine.

The experiment indicates that infection takes place relatively early in life and that in the main the disease encountered in the herd is a relatively chronic one, but the location and general type of reaction encountered in the acute disease is identical with that manifested in cases of longer standing. Furthermore the negative results obtained by the examination of two calves originating from the same source and born about the same time as the calves used in the experiment strengthen the argument that infection is acquired from the spontaneous infection in the older calves and that the disease may be maintained by the introduction of susceptible individuals.

Inasmuch as the indications pointed to the vibrios as the etiological agent it seemed desirable to test their pathogenicity on calves and the following experiment covers this phase of the problem.

Experiment 2.—Calf 1714 when 16 days old was fed three 48 hour blood agar cultures of vibrios obtained from the middle jejunum of Calf 1707. The cultures had been under cultivation for 3 months and during this period had been transferred eleven times. Symptoms indicative of enteritis, other than a slight general depression 2 days subsequent to feeding the culture, were not noted. When the calf was slaughtered 7 days after artificial infection a mild gastrointestinal catarrh was found. The principal involvement was in the upper and middle jejunum and irregularly scattered areas in the ileum where the mucosa was swollen and congested. Vibrios were obtained from the upper jejunum but the primary cultures from the middle jejunum and ileum were overgrown with *B. coli* and vibrios were not obtained from these regions. The liver was sterile.

On the whole the reaction was mild. The intestinal process seemed to be regressing but the organism was recovered. It seemed possible that the culture might have lost some of its virulence on cultivation, so that it was decided to test the pathogenicity of a freshly obtained strain and for this purpose the vibrios obtained from Calf 1714 were chosen.

Calf 1721 when 18 days old was fed six 72 hour blood agar cultures of vibrios obtained from the jejunum of Calf 1714. The cultures were of the third transfer and the strain had been under cultivation for 3 weeks. There was an increase in bodily temperature for the first 2 days following artificial infection and during this time the feces became dark, soft, and fetid. These were the only clinical manifestations noted except that the feces throughout the observation always contained large quantities of thick yellow mucus. In certain instances this material made up about half the stool. Microscopically it was composed of thick mucus loaded with leucocytes and epithelial cells. On the 5th day the feces became much softer and the animal was slaughtered.

In contrast to the ill defined clinical manifestations there was a severe inflammation of the small intestine which began at the pylorus and extended throughout the duodenum, jejunum, and the anterior half of the ileum. The intestinal walls were thickened, the vessels of the serosae injected, and the mucosa swollen, wrinkled, and bright red in color. The content was slimy and below the middle jejunum bile-stained. The liver appeared normal.

Many cultures of vibrios were obtained from the washed mucosa of the upper and middle jejunum and from the middle ileum but below this point the tubes were overgrown with *B. coli*. The tubes inoculated with bits of liver remained sterile. Lactose agar plate cultures revealed small numbers of *B. coli* in the content of the middle ileum and large numbers below this point. No *B. coli* and relatively few other colonies developed on the plates filmed with content from the duodenum and jejunum.

Both calves fed cultures of the vibrios manifested relatively slight clinical manifestations, but the organisms were nevertheless recovered from inflamed portions of the bowel. The inflammatory process in both instances resembled that found in the spontaneous disease and those cases which contracted the natural infection under experimental conditions.

DISCUSSION

A disease affecting calves from 18 to 68 days old has been described. In general it may be said that the clinical picture, other than a general condition of malnutrition and unthriftiness often accompanied by attacks of diarrhea and an irregular, moderate increase in bodily temperature, is confusing. Our data indicate that infection may take place relatively early, but the animals manifest at first only slight clinical manifestations, the symptomatology becoming more pro-

nounced as the disease becomes more chronic. That such animals may become a ready prey to other infections must be recognized. Our first cases seemed to be in general of this class and it was assumed that the intestinal disease had been superimposed on other conditions. The experiment in which young vigorous calves were exposed to older calves regarded as chronic cases of enteritis indicated that such infections may be acquired relatively early and may for a time be overlooked. A further argument that the disease is an independent one, and as such is not dependent on a preexisting one, is that calves may actually die as the result of such infection. Under certain conditions when the malady becomes chronic the clinical effects become so pronounced that slaughter is advisable. In one large herd many calves between the ages of 1 and 2 months have recently been slaughtered because of this disease.

The distribution of the lesions is characteristic. The upper half of the jejunum seems to be the most frequent locus, but inflammation of the mucosa of the abomasum is not uncommon and inflamed patches often of considerable size are frequently encountered in the upper, middle, and lower ileum. The process is one of congestion, loss of superficial mucosa, infiltration of the mucosa with leucocytes and round cells, and edema and congestion of the submucosa. The exudate overlying the inflamed mucosa and appearing in the feces is mucopurulent.

The etiology seems clear; the vibrios which were cultivated from the more chronic spontaneous disease, and from the young calves contracting the infection in a natural manner, all appeared alike in morphology and were all actively motile. In addition they all possessed certain well defined nutritive requirements. At times they could be readily demonstrated in films of the inflamed mucosa and were frequently found in many cultures from various portions of the involved intestine. However, in cases of long standing they are not easily obtained, since *B. coli* and other intestinal forms thrive readily in the content and serve to suppress the growth of the delicate vibrios in the test tube. In three instances they were cultivated from the liver. Experimental proof exists that vibrios that have been under cultivation for considerable periods still possess the property of passing from the mouth through the stomach and localizing in the small intestine and there inciting a process resembling that found in the spontaneous infection.

SUMMARY

An intestinal disorder of calves has been described. The clinical manifestations are usually observed in calves 2 or more weeks old. Our experiments indicate that infection may take place relatively early in life and may for a time produce only a mild reaction, but as the disease becomes more chronic the clinical manifestations become more pronounced. The anterior portion of the jejunum is the primary locus of infection but in more chronic cases practically the whole small intestine may be involved. Vibrios were cultivated from the inflamed intestinal mucosa in both the acute and more chronic spontaneous cases. Vibrios were also obtained from the acutely involved intestine of young calves experimentally exposed to natural infection. On three occasions similar vibrios were found in cultures from the liver. When a single strain of vibrios which had been under cultivation for 3 months was fed to a young calf subclinical infection was produced and the organism recovered. This strain after three passages on media when fed to a calf produced a severe inflammation of the jejunum and ileum and from these areas the organism was recovered.

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VIBRIOS (VIBRIO JEJUNI, N.SP.) ASSOCIATED WITH INTESTINAL DISORDERS OF COWS AND CALVES

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PLATE 31

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Although many vibrios were described following the discovery of the cholera organism relatively few have been shown to be pathogenic for man or animals.

Gamaléia (1) obtained vibrios (*V. metchnikovi*) from the blood and intestines of fowls suffering from enteritis. MacFadyean and Stockman (2) succeeded in cultivating vibrios from abortion disease in sheep and succeeded in reproducing the disease by injection of pure cultures into pregnant ewes. Since then such organisms have been obtained from sheep by Carpenter (3), Welch and Marsh (4), and Graham and Throp (5). Theobald Smith (6) isolated from the diseased placentas and aborted fetuses of cows vibrios (*Vibrio fetus*) which on injection into pregnant cows produced placental disease and abortion. In a series of papers (7, 8, 9) the disease and the etiological agent were discussed. A similar organism had been recognized by McFadyean and Stockman (10) and has subsequently been found to affect cattle in various parts of the world.

In addition to the disease-producing type of vibrios, Smith and Orcutt (11) reported vibrios obtained from the livers of young calves which resembled *V. fetus* in morphological and cultural characters but failed to agglutinate with sera specific for *V. fetus*.

The Source of the Cultures

The vibrios in this study were obtained from several sources but all were of bovine origin. The first three, 1629, 1641, and 1655, presumably originated from cases of infectious diarrhea (12) experimentally reproduced by feeding calves feces from spontaneous cases. The second pair comprises Vibrios 1669 and 1686 which were cultivated from the intestinal tracts of calves fed Cultures 1629, 1641, and 1655. The third group is composed of Vibrios 1652, 1699, 1700, 1728, 1731,

1745, and 1748, all obtained from spontaneous enteritis of calves (13). The fourth series includes Cultures 1707, 1709, 1714, and 1721 from enteric cases in calves experimentally produced, and finally Cultures 1208, 1522, 1524, and 1679 kindly furnished to us by Dr. Smith. *Vibrio fetus* 1660 was also furnished by Dr. Smith.

Method of Obtaining Vibrios from the Intestinal Tract

Two methods have been employed throughout the work. The less reliable may be said to consist in the inoculation of the water of condensation of veal infusion agar, to which five drops of defibrinated horse blood have been added, directly with the intestinal content of inflamed segments of the intestine. Immediate inoculations from the primary culture to secondary and tertiary tubes of the same media with the sterile loop will at times give pure cultures in one tube but overgrowth of intestinal bacteria is apt to occur and vibrios fail to develop.

A more complicated procedure but one on the whole more satisfactory is the following:

The seared serosa is cut, the intestinal tube flattened, and the content gently brushed from the inflamed mucosa. Four or five bits of the superficial mucosa, 1 to 2 mm. in diameter, are removed and washed in five or more changes of sterile salt solution. The mucosa is then ground in a Hagan (14) grinder, a little broth added, and the suspension inoculated by means of a loop into the condensation fluid of blood agar. It is customary to set up three series of two tubes. The first tube of each pair receives 1, 2, and 3 loopfuls of suspension, and after flaming the loop the secondary tubes are inoculated with 1, 2, and 3 loopfuls of the condensation fluid from the primary cultures. All tubes are then sealed with sealing wax and incubated. After 3 or 4 days tubes which show relatively little growth are examined microscopically. Even in spite of prolonged washing in many instances *B. coli*, streptococci, etc., may develop in the primary inoculations, but the secondary tubes are apt to contain only vibrios. In some cases vibrios will be found only in the first tube and then mixed with other organisms from which they can with difficulty be separated. In other instances only vibrios develop in the first of the series and the secondary tubes may or may not contain them.

In early cultures relatively little is manifested in the gross in the blood agar tubes. The condensation fluid is slightly turbid within 4 or 5 days and the blood's buffy coat is thickened. Later delicate lines at the border of the agar become visible and after several transfers

the border lines become well defined and a delicate film may spread from the condensation fluid to the lower slant. After prolonged cultivation many strains grow readily on the surface of the slant. From blood agar, cultures may be established in plain agar or in leptospira medium to which a little fresh kidney has been added. After prolonged cultivation on plain agar growth may even be obtained in broth where a heavy tenacious mass is formed on the bottom of the tube.

Certain strains grow well from the start, others adapt themselves slowly. In serum agar shake cultures the growth is on the surface or in a narrow zone just beneath the surface. In leptospira medium the heaviest growth is just beneath the surface (Fig. 1). From these facts it may be inferred that the organisms are not anaerobes.

When some of the condensation fluid of young blood agar is examined by means of the hanging drop actively motile elements are readily detected but it is difficult to ascertain their morphology because of their extremely rapid movement. Three types are frequently visible in the same culture. Short, slightly convoluted forms (Figs. 2 and 3) are the most active, and in suitable preparations one or two flagella situated at the poles may be demonstrated (Figs. 3 and 4). Forms with two or more complete coils as illustrated in Figs. 5 and 6 move more slowly and revolve about the long axis. The extremely long type as encountered in Fig. 7 is rarely motile although the body appears to sway. As the cultures become older clumps occur and cellular fragmentation or granule formation is the rule. In Fig. 5 the edge of a clump of granules resulting from vibrionic degeneration is illustrated, and several reveal early degenerative changes, and in Fig. 7 an atypical granule formation is visible.

The cultures stain well with ordinary stains after a longer time than is usually required. In films prepared from tissues only prolonged staining seems to color them but in fresh, unstained preparations of tissue and exudate they may be readily recognized. They are Gram-negative. The optimum reaction for growth is about pH 7.6. The organisms fail to grow in either slightly acid or definitely alkaline media. Cultures soon die when dried in the air and a temperature of 55°C. for 5 minutes kills them. When autoclaved moist cow feces were inoculated with vibrios and stored in the room the vibrios lived

for 6 days but when the feces became thoroughly dried cultures could no longer be obtained. Distilled water is not especially toxic since actively motile forms may survive for 24 hours. The vibrios resist the soluble action of bile.

Pathogenicity for Laboratory Animals

In accompanying papers we have described (12, 13) our experiments dealing with the pathogenic properties of these organisms for cows and calves. It can be said that for laboratory animals the organisms possess little pathogenicity by the usual standards. Guinea pigs and white rats are refractory when injected intraperitoneally. White mice are more susceptible since two of the strains (1655 and 1686) when injected in small quantities intraperitoneally produced multiple necrotic foci of the liver from which the vibrios were cultivated. Other cultures failed to do so.

Strains 1629 and 1641 possessed no pathogenicity for rabbits, but others (1669, 1686, 1731) when freshly obtained and injected intravenously produced well marked febrile reactions and during this phase vibrios were readily cultivated from the peripheral blood and organs. After the fever declined there was established a catarrhal inflammation of the small intestine, at times accompanied by diarrhea. The vibrios were recovered from the inflamed intestinal mucosa. At this stage the blood and organs were sterile.

Feeding culture to rats resulted negatively. In one mouse of ten fed various cultures vibrios were obtained from the small intestine. Vibrios fed to unprepared rabbits fail to establish themselves in the intestinal tract but when 0.5 gm. of sodium bicarbonate is administered a brief interval before the culture is introduced into the stomach, the vibrios reach the small intestine where they produce catarrhal inflammation and can be recovered after 5 days.

The Immunological Relationship of Various Strains as Judged by Agglutination

It was decided to correlate by means of agglutinin the vibrios obtained from epidemic diarrhea in cows and enteritis in calves with those obtained by Dr. Smith, and with this in view a number of rabbits were immunized with living cultures.

Inasmuch as the cultures grow relatively poorly it was a considerable undertaking to obtain sufficient antigen from plain agar growths for the tests. If, however, to tubes 2.5 cm. in diameter containing 10 to 12 cc. of melted veal infusion agar a fragment of guinea pig or rabbit kidney is added, the tube slanted, and later heavily seeded with young culture and sealed with sealing wax, excellent growth is obtained after 3 or 4 days incubation and suspensions of such growth in NaCl solution afford good antigens.

In Table I the results of the agglutination tests are shown. The same form of arrangement as indicated on page 587 has been followed. The higher serum dilutions in the tests with *Vibrio fetus* antiserum have not been shown since this serum fails to agglutinate the heterologous strains at dilutions higher than those shown.

It is clear from Table I that there exist among the intestinal vibrios at least two well defined immunological groups. Two of the strains presumably originating from cases of infectious diarrhea in adult cows (1629 and 1655) agglutinate well with the same serum and when the serum is absorbed with the heterologous strain the agglutinins are equally removed from both organisms.

The large group, which comprises all but one of the calf enteritis group, one of those presumed to have originated from diarrhea in cows, all the vibrios cultivated from the experimentally induced disease, and the cultures given to us by Dr. Smith, forms what appears to be one immunological group. Vibrio 1728 is not agglutinated by any serum.

The serum specific for *Vibrio fetus* Culture 1660 agglutinates slightly many of the cultures in only the lower dilutions.

Although the members of the large group were strongly agglutinated by both Serums 1641 and 1700 it has been possible to show by absorption that certain vibrios possess a more complex antigen. For instance, if Serum 1641 was absorbed with the homologous strain agglutinin was removed for all strains. If, however, Culture 1700, which was strongly agglutinated by Serum 1641, was used for absorption the agglutinin still remains for the homologous organism and a number of others. This is brought out in Table II.

If Culture 1700 possessed only part of the antigen of strains behaving like 1641, then its antiserum should be completely absorbed by organisms possessing the complete antigen. This proved to be correct. Table III indicates the results of the agglutination tests of Serum 1700

TABLE I

The Agglutination Affinities of Intestinal Vibrios

No. of culture	Source	Antiserum Culture 1629 — Serum dilutions								Antiserum Culture 1641 — Serum dilutions								Antiserum Culture 1700 — Serum dilutions							Antiserum Culture *Vibrio fetus* 1660 — Serum dilutions		
		1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:80	1:160	1:320
1629	Presumably originating from diarrhea in cows	C	C	C	C	C	+++	++	+	++	+	+	+	±	–	–	–	–	–	–	–	–	–	–	–	–	–
1641		–	–	–	–	–	–	–	–	C	C	C	C	C	C	++++	+++	C	C	C	++++	+++	++	+	++	+	±
1655		C	C	C	C	C	C	+++	++	+++	++	++	+	–	–	–	–	+	+	–	–	–	–	–	++	+	–
1669	Recovered from calves fed Cultures 1629, 1641, and 1655	–	–	–	–	–	–	–	–	C	C	C	+++	+	±	–	–	C	C	C	C	+	±	–	–	–	–
1686		–	–	–	–	–	–	–	–	C	C	C	+++	+	±	–	–	C	C	C	++++	+	+	±	–	–	–
1652	Obtained from spontaneous enteritis in calves	–	–	–	–	–	–	–	–	C	C	C	C	C	C	++	+	C	+++	++	++	++	±	–	++	++	–
1699		–	–	–	–	–	–	–	–	C	C	C	C	+++	+	±	–	C	C	C	C	++	+	±	–	–	–
1700		–	–	–	–	–	–	–	–	C	C	C	C	+++	±	–	–	C	C	C	C	++	±	–	+	–	–
1728		+	+	+	±	–	–	–	–	±	±	–	–	–	–										++	+	+
1731		–	–	–	–	–	–	–	–	C	C	++++	+++	++	+	–		C	C	C	++++	++	±	–	+	±	–
1745		–	–	–	–	–	–	–	–	++++	++++	++++	++++	+++	++	++	+	C	C	++++	++	++	+	±	++	+	+
1748		+	+	–	–	–	–	–	–	+++	+++	++	++	+	–	–	–	+++	+++	+++	++	++	+	–	++	+	±
1707 Intestine	Recovered from naturally induced and artificially infected cases of enteritis in calves	–	–	–	–	–	–	–	–	C	C	C	C	C	C	++++	+++	C	++++	++++	+++	++	–	–	++	+	±
1707 Liver		–	–	–	–	–	–	–	–	C	C	C	C	C	C	+++	+	C	C	C	++++	++	+	–	++	+	–
1709		–	–	–	–	–	–	–	–	++++	++++	+++	++	+	±	–	–	++++	++++	++++	+	±	–	–	+	–	–
1714		–	–	–	–	–	–	–	–	C	C	C	C	C	C	++++	+++	C	C	++++	+++	++	+	–	+++	+	–
1721		–	–	–	–	–	–	–	–	C	C	C	C	C	C	+++	++	C	C	C	++++	++	+	–	+++	+	±
1208 Liver	Obtained by Dr. Smith from calves	–	–	–	–	–	–	–	–	C	C	C	+++	+	±	–	–	C	C	C	C	+++	±	–	+	–	–
1522 Liver		+	+	±	±	±	–	–	–	C	C	C	++++	++	++	–	–	C	C	C	C	C	++	+	++	+	±
1524 Liver		–	–	–	–	–	–	–	–	C	C	C	C	C	C	++	+	+++	++	++	+	–	–	–	+++	+	+
1679 Lung		–	–	–	–	–	–	–	–	C	C	C	++++	+++	+++	++	–	C	C	++++	+++	++	+	±	++	+	±
1660 *Vibrio fetus**	Obtained by Dr. Smith from case of vibrionic abortion	±	–	–	–	–	–	–	–	+++	++	+	–	–	–	–	–	+++	+++	+	±	–	–	–	C	C	C

* *Vibrio fetus* in homologous serum had the following titer: 1:640 ++++, 1:1,280 ++, 1:2,560 +, 1:5,120 –

which had been absorbed with Culture 1641. In the experiments the serum was tested at dilutions as great as 1:20,000 but agglutination

TABLE II

The Effect of Absorption of Serum 1641 with Culture 1700

No. of culture	Dilutions of serum							
	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
1641	C	C	C	C	C	C	++++	++
1700	+	−	−	−	−	−	−	−
1208	++	+	±	−	−	−	−	−
1652	++++	C	C	C	C	C	++++	+++
1699	++	+	+	+	+	−	−	−
1707 Intestine	C	C	C	C	C	++++	++	++
1707 Liver	+	+	±	−	−	−	−	−
1709	+++	++	++	+	−	−	−	−
1714	C	C	C	C	C	C	++++	+++

TABLE III

The Effect of Absorption of Serum 1700 with Culture 1641

No. of culture	Dilutions of serum				
	1:80	1:160	1:320	1:640	1:1,280
1641	++	+	±	−	−
1700	+++	++	+	±	−
1208	++	+	+	−	−
1652	++	++	+	±	−
1699	++	+	±	±	−
1707 Intestine	++++	++++	+++	+	−
1707 Liver	+++	++	+	−	−
1709	++	+	±	−	−
1714	++	+	±	−	−

stopped at the titer of 1:640. The higher dilutions have been omitted from the table.

The experiment was repeated but this time 1641 was absorbed with

Culture 1707 which, from the data in Tables II and III, was considered to comprise the complete antigen. The results indicated that Culture 1707 contained the complete antigen since the titer of the serum for all vibrios was greatly reduced.

It is true that the vibrios with the exception of one strain fall into two well defined groups, the smaller comprising only two strains which both originated from epidemic diarrhea in cows, the larger embracing the vibrios cultivated from the inflamed intestinal tracts or livers of calves. The latter group may be divided on the basis of agglutinin absorption into two types, one possessing a complete antigenic character and the other possessing only a portion of the antigen. It is also true that none of the vibrios are antigenically similar to the culture of *Vibrio fetus*.

DISCUSSION

The vibrios while presenting certain slight morphological differences, such as the length, the number of coils, and to some extent the depth of coils, nevertheless resemble each other sufficiently to be regarded as a closely related group. Since their first locus in the intestinal tract as judged by their presence in lesions encountered in acute infections is the jejunum, the name *Vibrio jejuni* is proposed.

Their pathogenic properties for cows and calves have been discussed in earlier papers. In the main the disease induced resembles to a certain extent human cholera and the vibrios from the bovine disease often resemble in young cultures the human organism. Both maladies are infections of the small intestine and both are characterized by excessive mucous secretion. However the bovine vibrios differ markedly from the comma vibrio in cultural characters. The bovine group are more difficult to grow, fail to liquefy gelatin or blood serum, and will not survive on strongly alkaline media. Thus far they have not been shown by means of acid or gas production to utilize carbohydrate. Similarities in pathogenic properties for rabbits exist, both organisms when introduced into the circulation produce fever and penetrate the intestinal wall. In both cases it is necessary to neutralize the acidity of the stomach to infect rabbits by mouth. No such procedure is necessary in infection experiments with the cow or the calf since the vibrios readily pass the stomach and frequently are observed in the

fecal mucus after 36 hours. It should be stated that the cultures recovered from the livers of artificially infected mice, and from the peripheral blood, organs, and small intestines of rabbits were proved by agglutination to be similar to those injected.

The grouping according to agglutination affinities has been of assistance to us in certain respects. When Culture 1707, which had been shown to possess the whole antigen of the larger group, was fed to Calf 1714 it gave rise to definite intestinal disease and the vibrio obtained from the jejunum also possessed the whole antigen. When Culture 1714 was fed to Calf 1721 more severe disease was encountered and Vibrio 1721 was shown to possess the complete antigen. Evidently the character is transmitted in spite of considerable variations in the environment. That both types may exist in cultures from different regions in the same animal is also true. The vibrios obtained from the intestine of Calf 1707 possessed the whole antigen while the strain cultivated from the liver failed to possess the complete complex. This has been true in other instances, all the vibrios from the liver studied have failed to contain the complete antigenic complex.

In regard to the relation of the vibrios originating in the intestinal tract to *Vibrio fetus* all that can be said is that they failed to show the same agglutination affinities as the culture of *Vibrio fetus* employed.

SUMMARY

A number of vibrios obtained from the small intestines of calves fed feces from spontaneous diarrhea in cows, natural intestinal disorders of calves, experimentally induced infections of calves, and cultures obtained from Dr. Theobald Smith have been studied. From the close morphological resemblance, similarities in motility, position and number of flagella, tinctorial properties, and the tendency to fragmentation in older cultures, as well as the narrow nutritive requirements, we are led to regard them as a closely allied group and we propose the name *Vibrio jejuni*.

Immunologically as judged by agglutination the organisms have been divided into two groups, the smaller representing two strains originating from diarrhea in cows and the larger comprising one from this source and many from the calf disease. The larger group can be

subdivided by means of agglutination absorption into cultures which do not contain the complete antigenic complex and others which do so.

Certain freshly isolated vibrios when injected into rabbits incite definite reactions terminating in a localization in the small intestine accompanied by catarrhal inflammation.

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EXPLANATION OF PLATE 31

FIG. 1. Vibrios 1629 and 1641. Seven days growth on leptospira medium and guinea pig kidney about natural size.

FIG. 2. 3 day plain agar culture Vibrio 1700, 40th transfer, illustrating slightly convoluted forms. Dilute carbolfuchsin. × 1,500.

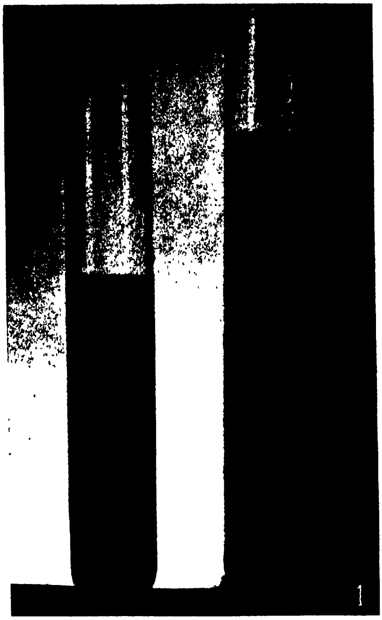
FIG. 3. Young plain agar culture Vibrio 1629 showing flagella. Bailey's flagella stain. × 1,400.

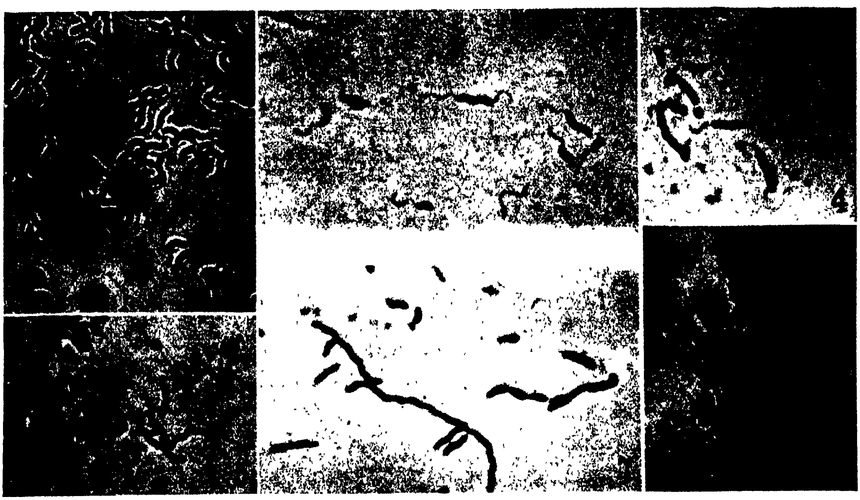
FIG. 4. Young plain agar culture Vibrio 1700. Bailey's flagella stain. × 1,400.

FIG. 5. 3 day blood agar culture Vibrio 1629, sixth transfer, showing longer coiled forms and a portion of a clump in which the vibrios have fragmented. Giemsa stain. × 1,500.

FIG. 6. 4 day blood agar culture Vibrio 1655, first transfer. Long and short coiled forms are illustrated. Giemsa stain. × 1,500.

FIG. 7. 3 day plain agar culture Vibrio 1629, 35th transfer, showing extremely long forms as well as shorter types and illustrating early fragmentation. Giemsa stain. × 2,000.





(Jones *et al.*: Vibrios and intestinal disorders of cattle)

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GENETIC NON-DISJUNCTIONAL FORMS IN DROSOPHILA

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During the past thirteen years the writer has been interested in a stock producing forms which differ from the normal in a variety of ways, but chief among which is the enlarged pattern of the ommatidia of the eye. The abnormal flies vary within themselves. Some have a coarse eye pattern and a slightly enlarged size throughout as the chief characteristic by which they may be separated from the normal. Another group shows changes in the sex organs and secondary sexual characters, another group has the wings cut and the wing veins irregular, the fly giving a wholly debilitated appearance. A further type appears as a small fly with slender short bristles. The appearance of these types suggests the individuals which Bridges has described as triploids, sex-intergrades, supersexes and haplo IV. They differ from his material, however, in their much more frequent appearance within this stock, where all types of these individuals may appear as often as once in thirty-five times. They further differ from his material in that the abnormal forms are due in last analysis to the action of a single gene. The purpose of this paper is to describe the anatomical structure of these different forms and to compare them with the normal.

The Normal

Fig. 1 shows the normal male and female *Drosophila* used as parents for the production of the atypical offspring.

The most important points of difference between the sexes visible in Fig. 1 are: the male is distinctly smaller than the female; the male has a black comb-like structure (sex-comb) on one of the joints or tarsi of the front legs, whereas these structures are lacking in the female.

The abdomen of the male presents a less banded appearance than that of the female due to the difference in the distribution of pigment, although both are segmented. The external genitalia of the male are composed of plates of a shape very different from those of the female. These are known as the clasper plates and the anal plates. Those of the female are called the egg guide and anal plates. The relative size of the facets composing the compound eye is shown in the camera lucida outline of one facet.

The internal anatomy is equally distinct. The male had the following organs: two yellowish coiled tubes, the testes; two glandular structures, the paragonia, opening into the vas deferens, a duct leading from the testes and paragonia to the ejaculatory sac; the sperm vesicle,

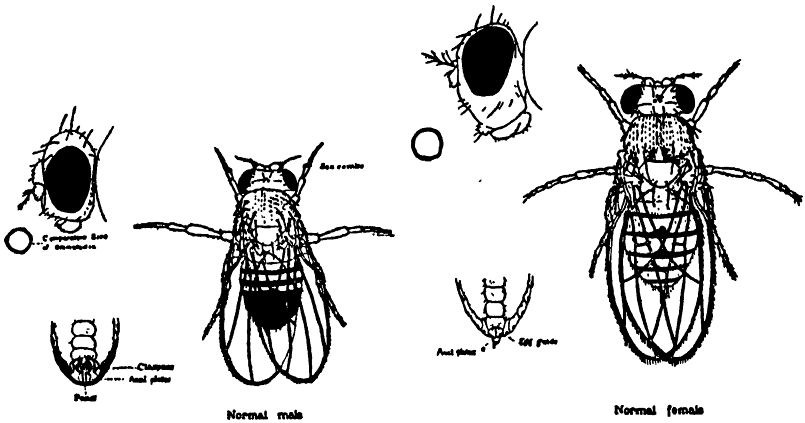


FIG. 1. The normal male and female *Drosophila melanogaster*.

ejaculatory sac; the ejaculatory duct leading from the sac to the penis; and the penis. The female organs are: the ovaries; ducts leading from each ovary and joining into a common duct, the oviducts; the uterus; two mushroom-shaped dark-colored organs, spermathecae, with ducts opening into the dorsal wall of the anterior end of the uterus and containing sperm after copulation; another receptacle for sperm known as the ventral receptacle; the parovaria; and vaginal portion of the uterus. The male and female flies which are the parents of the abnormal chromosome type appear to be entirely normal in respect to all these organs.

The Variant Types

Within such a group of normal individuals coming from normal parents, there occasionally appears a fly which is a variant from type in one or many particulars. The changes may affect any or all of the organs mentioned. The affected flies are generally sterile. The sex instincts are altered, the duration of life materially shortened. The first general group are individuals which have within themselves both male and female organs. These sex-intergrades may be more or less arbitrarily divided into three general types. Figs. 2, 3 and 4 show certain type-forms selected to show the extent of the changes in the

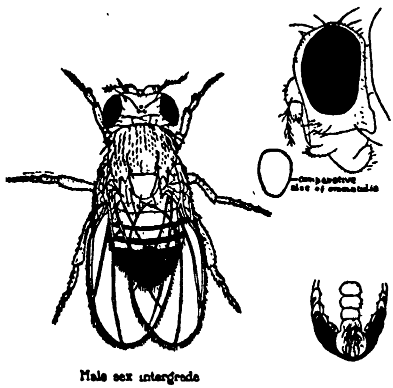


FIG. 2. Male sex-intergrade. This fly is seen to resemble closely the male externally. It differs chiefly in the pattern made by the facets of the compound eye which are large and therefore make the grid appear bigger. The outline of the facet size should be compared with the normal male as both are drawn to the same scale.

external organs. These forms may be arranged arbitrarily from those most like the male to those most like the female. Fig. 2 shows the male type in appearance, the only striking change being the enlarged grid of the eyes formed by the increased size of the facets. The relative size of these facets is shown by the camera lucida outline of one of average size. It will be noted that the facets are much larger than those of the normal fly. Fig. 3 shows the intermediate type: the external appearance of the body being generally male, the external genitalia female, and the eyes with the enlarged grid. Fig. 4 shows the other extreme:—size of body and pigmentation of abdominal

segments are of the female type. The sex-combs are generally present. Eyes show the enlarged grid. The genitalia are female type.

The types of changes which are indicated are also frequently accom-

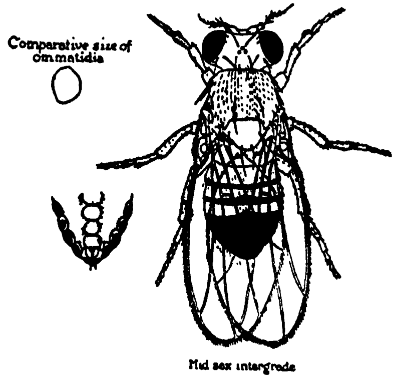


FIG. 3. Mid sex-intergrade. The noticeable features in the external appearance of individuals of this type are body appearance (sex-combs, size and pigmentation of abdominal plates) like the males while the genitalia are female. Eyes show the enlarged grid.

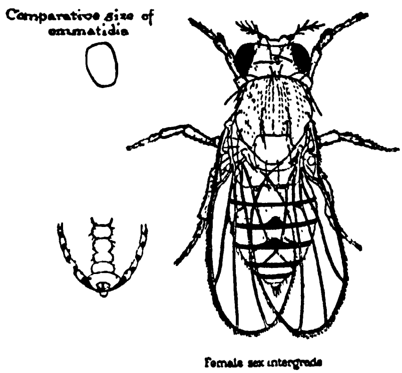


FIG. 4. Female sex-intergrade. The type of body is female with certain alterations toward the male: sex-combs and sometimes male genitalia. The eyes have the enlarged grid.

panied by profound changes in behavior, both the male and female type often losing their sex instincts. These flies are uniformly sterile whether they resemble males or females in appearance. While the

abnormal animals have been described as type specimens, it should be understood that all intergrades between normal males and females are to be found. They are sex-intergrades in the same sense as those described by Bridges.

Besides these sex-intergrades there is another abnormal type. These flies are generally larger than the ordinary females. They are entirely normal females in appearance save the grid on the eye which is enlarged. They are fertile but are capable of reproducing markedly different progeny than those of type males and females. These progeny are of the following kinds; normal males and females, sex-intergrades of the three types described and females like themselves. These females are triploids.

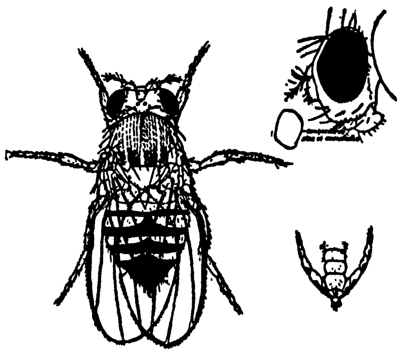
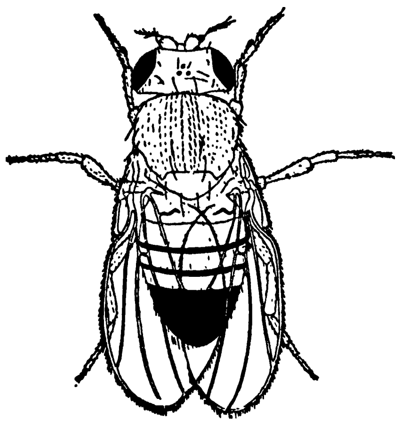


FIG. 5. Female type of fly having enlarged grid in eye pattern. Fly breeds as female reproducing normal males and females and a great excess of abnormal types shown in Figs. 2, 3, 4 and 5.

Another type of fly with very fine eye grid and bristles is less frequently found among the progeny of these normal-appearing parents within this line of flies. These flies are physically weak and show a short duration of life. They may be of either sex. Both sexes are frequently sterile and when fertile are of low productivity. Fig. 6 shows a male of this type.

The last type of fly found less frequently than the preceding types has a forlorn appearance, wings cut, wing veins thickened and irregular, eyes bulging with a somewhat enlarged grid, body shrunken and female throughout. Duration of life is short. These flies are sterile.

In the sex-intergrades, the internal changes are more extensive than the external changes. The male-appearing sex-intergrades tend to have organs of more nearly the male type, the midsex-intergrades the most pronounced abnormalities, and the female sex-intergrades tending to be more nearly like the females. Thus parents 134 had a male type of sex-intergrade among their offspring. This male sex-intergrade showed a more or less typical male appearance—sex-combs, anal plates and clasper plates. The wings were cut out, a fairly frequent change in the body of such animals. The paragonia, vas deferens, ejaculatory sac, penis and testes were present. The left



Slender bristles. male

FIG. 6. Male showing slender bristles and fine eye grid.

testis was very small and intensely yellow. The egg guide, spermathecae, ovaries, parovaria, uterus or ventral receptacle of the female were not present.

Fig. 7 shows a drawing of the sex organs of the male sex-intergrade type. One testis is fairly normal, the other much reduced in size and of abnormal shape resembling more nearly the testis as found in the pupa. Only one paragonium of reduced size was present. Vas deferens, ejaculatory sac and penis somewhat reduced in size. Clasper plates present. Flies of this type are sterile.

In the mid sex-intergrade class, which is after all only the extreme of either class, was a fly from Female 132 which showed a general male

appearance so far as the distribution of pigment on the abdominal segments was concerned. Sex-combs were present. The anal plates, egg guide, two spermathecae, ovaries (left one very small, largely trachea), uterus and ventral receptacle of the female were present while the parovaria were absent. Of the male internal organs only a much deformed, small, yellow, right testis was present. In another such case from Female 539, the general appearance of the segments and body was female. The fly showed the following male organs: sex-combs, anal plates; one juvenile, yellow testis and one long, wrinkled, thin testis; vas deferens; and ejaculatory sac. The female

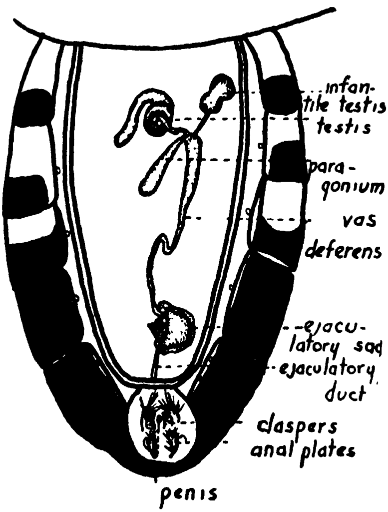


FIG. 7. Sex organs of male sex-intergrade.

organs present were egg guide, two spermathecae, parovaria, uterus and ventral receptacle. The male organs lacking were clasper plates, paragonia and penis. The female organs lacking were the ovaries.

Fig. 8 shows certain of the changes found in flies of the mid sex-intergrade type. What appeared to be a testis of the infantile type was present. No ducts or other male organs were present save the pigmentation of the abdominal plates, which was male in type. Anal plates were much deformed, clasper plates present. Two spermathecae and a ventral receptacle were present although much reduced in size. Uterus and female genitalia were present.

In the female sex-intergrade class there is somewhat less uniformity than in the male class. Thus one sex-intergrade from Female 102 had a general female appearance, so far as body and pigmentation of the segments were concerned. Sex-combs were present. Anal plates were much deformed, clasper plates present. Two small ovaries chiefly composed of tracheal tissue were present. Other normal organs of both sexes were absent. Another case from Female 133 had quite a normal female appearance, no sex-combs, wings cut, female anal plates, egg guide, two spermathecae, two ovaries of somewhat reduced size, parovaria, uterus and ventral receptacle. No male

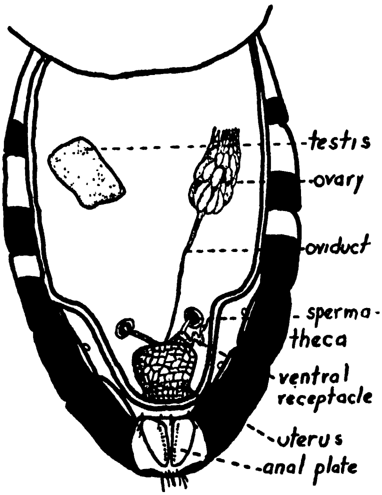


FIG. 8. Sex organs of a mid sex-intergrade. This figure should be compared with Fig. 6.

organs were present. In every case the eyes showed the enlarged grid.

Fig. 9 shows the generative organs as found in a female sex-intergrade. Only one ovary of much reduced size was present. The oviduct was small. Two spermathecae of small size present. The parovaria and ventral receptacle were absent. Uterus and external female genitalia present. The abdominal plates were of the female type.

A table, showing in somewhat diagrammatic form the changes found in a number of cases, is given. Histological examination of many of

this type of flies frequently shows testicular and ovarian tissue present in the same organ.

The abnormal type shown in Fig. 5 has the female organs throughout. The only apparent difference between these organs and those of the normal female is that they give the impression of being slightly larger.

Flies of the type of Fig. 6 are either male or female throughout, no blending of the characters taking place. The sex organs are in general reduced in size, the flies frequently being infertile.

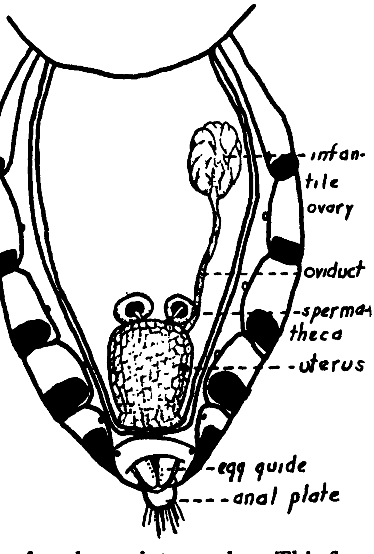


FIG. 9. Sex organs of a female sex-intergrade. This figure may be compared with Figs. 6 and 7.

The last type of fly is female throughout and sterile. The ovaries and oviducts seem shrunken in appearance. Sections of the ovaries are atypical.

In their broader aspects the specimens described present a rather definite pathological picture, involving changes in the cell size, reproductive organs, etc. The group brought together as sex-intergrades are quite uniformly sterile. The type shown in Fig. 5 is fertile but shows its abnormal constitution in the fact that while breeding as a female progeny are produced which are of the normal and abnormal types.

TABLE I

Abnormalities in Structure of Case Specimens

Number	Sex-combs	General appearance	Wings	Anal plates	Egg guide	Clasper plates	Spermathecae	Ovaries	Parovaria	Uterus	Ventral receptacle	Testes	Paragonia	Vas deferens	Ejaculatory sac	Penis
							Organ examined									
6278	+	♂	+	♂	−	+	−	−	−	−	−	+	−	+	+	+
6278	+	♂	+	♂	−	+	−	−	−	−	−	left + right small	+	+	+	+
6279	+	♂	+	♂	−	+	−	−	−	−	−	+ small	+	+	+	+
6296	+	♂	+	♂	−	+	−	−	−	−	−	+	+	+	+	+
6296	+	♂	+	abnor-mal ♂	−	abnor-mal +	−	−	−	−	−	+	1 small +	+	+	+
6299	1 small +	♂ +	+	♂	−	+	+	very small	−	+	−	−	−	−	−	−
6297	+	♂	+	♂	−	small twisted −	−	−	−	−	−	1 very small +	+ 1 very small	−	−	−
6296	+	♂	+	♂	−	+	−	−	−	−	−	small +	+	+	+	+
6310	+	♂	+	♂	−	+	−	−	−	−	−	+	+	+	+	+
6324	+	♂	+	♂	−	+	−	−	−	−	−	+	+	+	+	+
134	+	♂	cut	♂	−	+	−	−	−	−	−	yellow small +	+	+	+	+
117	+	♂	+	♂	−	+	−	−	−	−	−	yellow +	+	+	+	+
112	+	♂	cut	♂	−	+	−	−	−	−	−	yellow small +	+	+	+	+
114	+	♂	+	♂	−	+	−	−	−	−	−	yellow +	+	+	+	+
171	+	♂	+	♂	−	+	−	−	−	−	−	+	+	+	+	+

TABLE I—*Concluded*

	Organ examined															
Number	Sex-combs	General appearance	Wings	Anal plates	Egg guide	Clasper plates	Spermathecae	Ovaries	Parovaria	Uterus	Ventral receptacle	Testes	Paragonia	Vas deferens	Ejaculatory sac	Penis
115	+	♂	+	♂	−	+	−	−	−	−	−	small yellow +	+	+	+	+
133	+	♂	+	♂	−	+	−	−	−	−	−	yellow +	+	+	+	+
133	+	Mid	+	−	−	−	−	−	−	−	−	small yellow +	+	+	+	+
133	+	♂	+	♂	−	+	−	−	−	−	−	yellow +	+	+	+	+
133	+	Mid	+	−	+	−	−	small left ovary	−	−	−	small yellow right testis	−	−	+	+
132	+	Mid	+	♀	+	−	+	small left +	−	+	+	small yellow right testis	−	−	−	−
132	+	♂	+	♂	−	+	−	−	−	−	−	+	+	+	+	+
133	−	♀	cut	♀	+	−	+	+	+	+	+	−	−	−	−	−
111	+	♂	+	♂	+	−	+	−	+	+	+	2 infantile	−	−	−	−
102	+	♀	+	♀	+	mixed −	−	+ 2 small trachea tissue	−	−	−	−	−	−	−	−
102	+	Mid	+	♂	+	−	+	1 small	−	−	−	1 small yellow	−	−	+	−
205	+	♂	cut	♂	+	−	+	−	−	+	+	2 small yellow +	−	−	−	−
539	+	♀	+	♂	+	−	+	−	+	+	+	2 small yellow +	−	+	+	−
924	+	♂	+	♂	−	+	−	−	−	−	−	+	1 small	+	+	+

A measure of the effect of these alterations from the normal may be had by studying the sum total effect as indicated by the time the individual's life mechanism is able to continue functioning. Experiments to determine the duration of life of each group have been performed and while of much interest in themselves need only be summarized here. The normal females live slightly longer than any other class. Individuals like those of Fig. 5 live a short time less than the normal females. The difference is not significant. The normal males live a somewhat less time. Individuals of the type of Fig. 2 have but half the duration of life of the previously mentioned classes and flies of the type of Fig. 4 live the shortest time of all, only one third the duration of life of the other classes. These differences are all markedly significant and are of such extreme proportions as 3 to 1.

The Chromosome Structure

In the type male and female of *Drosophila melanogaster* it will be recalled that there are four pairs of chromosomes. The male has an X and Y, two pairs of long V-shaped chromosomes, the II and III, and a pair of small round chromosomes, the IV pair when seen in the metaphase plate. The female has a pair of rod-shaped X-chromosomes, two pairs of V-shaped chromosomes and a small round pair.

The normal-appearing males and females of the stock producing the sex-intergrades, triploids, slender bristled flies and super-females have been examined for their chromosome structure. Drawings of typical plates are shown in Fig. 10.

The chromosome figures of the normal-appearing flies within this particular stock are seen in Fig. 10 to be essentially the same as those of the ordinary wild type stocks.

The female chromosomes differ from those of the male in that the female normally has two X-chromosomes and no Y-chromosome. Otherwise the female chromosomes II, III and IV are the same as those of the male. This is the most typical case of this particular stock. Occasionally the female cells show besides the two X-chromosomes a Y-chromosome like that of the male. This Y-chromosome appears to play no particular part in the further life of the fly; in fact, is simply a concurrent mechanical condition brought about by the atypical maturation processes of the egg formation. The normal

males and females of this stock are therefore essentially like the males and females of wild types.

The cells of the sex-intergrades, Figs. 2, 3 and 4, show a triploid instead of a diploid complex, there being twelve chromosomes present instead of eight, two X-chromosomes, a Y-chromosome and three each of the second, third and fourth chromosomes. The fourth chromosomes are not always clear in all the figures, as they are small and

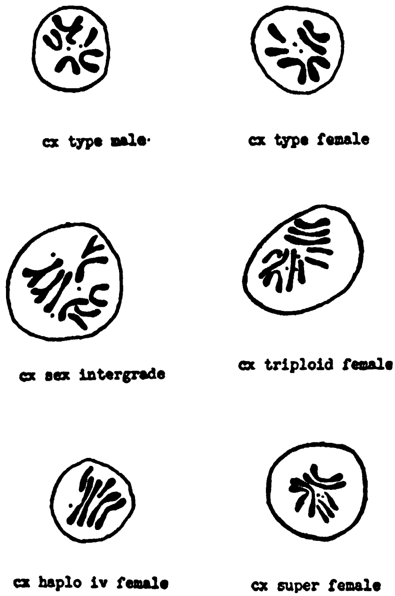


FIG. 10. The chromosomes of the male and female *Drosophila*, cx. stock, and sex-intergrades, triploid females, haplo IV's and superfemales appearing as progeny from them.

difficult to differentiate. Fig. 10 shows the chromosome plate of such a form.

The changes which are found in flies of the type of Fig. 5 are similar to those in the other forms. Fig. 10 shows the chromosome plate of such a fly. The chromosomes are in triplicate throughout: three X's, three II's, three III's and three IV's, with occasionally a Y-chromosome. This arrangement of the chromosomes enables this fly to form normal eggs and diploid eggs. The dual nature of the eggs from these flies results in a repetition in the progeny of the types previously

described, normal males and females, sex-intergrades and triploid females. This repetition of the different types in the progeny of such triploid females is proof that the abnormal chromosome number is the underlying cause which is responsible for the abnormalities found.

The slender bristled flies are found to have a normal chromosome constitution, save in one particular; they have only one fourth chromosome. The low viability of such flies makes this difficult of proof in every case, but both the cytological figures found and the genetic tests possible all substantiate this conclusion. They apparently correspond to Bridges' haplo IV.

The debilitated females are triploid in the sex chromosomes but diploid otherwise. They are apparently identical to super-females.

The Gene as the Etiological Agent

Since the purpose of this paper is simply to present the anatomical aspects of the problem, it need only be said that the physical entity causing these extensive changes, when viewed grossly or microscopically, is a gene in the middle of the third chromosome.

DISCUSSION

The sex-intergrades, triploids, slender bristled weak males and super-females, as herein described, come directly from normal parents. A single pair of these normal flies may give 1 to 6 abnormal flies during the course of the culture, seldom more than that. These atypical flies give the first inkling of the abnormal conditions in the maturation processes of the eggs within the parents of this particular strain. All these atypical forms have an abnormal chromosome number within each of their cells. The extensive alterations from the well-known wild-type fly are due to the fact that these mothers gave them eggs having their peculiar chromosome numbers. The flies may be considered as primary non-disjunctional forms in which we are observing the origin of new types. The types described by Bridges, since they come from and as a mechanical consequence of this primary non-disjunction, are secondary non-disjunctional forms. It is of interest therefore to note the similarity of these individuals with the secondary non-disjunctional triploids, sex-intergrades, super-females and minute bristled flies as described by Bridges and by Dobzhansky and Bridges.

So far as can be determined the types are identical, type for type, in their external appearance, internal structure and chromosome characteristics and even to the variability observed in the organ distribution of the intersexes.

It is, however, possible to obtain all types of non-disjunctional forms as primary individuals from this stock, whereas the secondary forms are less likely to be found all within one strain.

Within this strain the frequency of the formation of these primary non-disjunctional individuals varies from as low as 1 to 100 flies to as high as 1 to 30. This ratio is 100 to 300 times the rate found in ordinary stocks. Bridges has made the suggestion that when normal stocks have such an occasional primary non-disjunctional individual found in the progeny that such an individual comes from cells within the ovary which are markedly larger than the ordinary cells and carry more than the ordinary complement of chromosomes. This reasoning is based on the evidence that "in three separate preparations of ordinary 2 N females it was found that a portion of an ovary was constituted of markedly larger cells; and in two of the individuals some of the giant cells were in division, and the chromosomes could be counted as 4 N. Evidently there had been in some oogonial cell a division of the chromosomes that had not been followed by division of the nucleus and cytoplasm. The resulting tissue was tetraploid and any reduced gamete would be 2 N. Such a 2 N gamete, fertilized by a normal sperm, would account for each of the twenty-five recurrences of triploid." In view of the increased frequency of the abnormal forms within the strain here described it would be expected that, if the hypothesis of Bridges were true, areas of large-sized cells and multiple chromosome numbers would be quite common within the ovaries of females from this strain. Such is found not to be the case. No enlarged cells have been observed within the ovaries of the normal parents. Neither have cells been observed with increased chromosome numbers. The evidence of the number of abnormal types per parental bottle furthermore points rather to the maturation division as the point where the non-disjunction takes place in forming these atypical chromosome flies at least for this stock.

The data herein presented support the contention that sex is determined as an interaction of the autosomal chromosomes and the sex

chromosomes. The fact that within the intersex group there is such a wide variation in the degree with which the sex characters are developed and that this variation seems to be correlated with the type of gene complex found in the chromosomes further points to the conclusion that the gene elements within the chromosomes are the responsible factors and not the chromosome *per se*.

The cause of these changes is a third chromosome factor. To the extent that this factor modifies the end product of the reaction sex it could be looked upon as a sex factor. It is, however, probably better since it is one of the very few genes for which we have some information of its physical action to regard it rather as it really is—a factor whose presence or absence materially influences the maturation of the chromosomes within the female.

The cell size of the ommatidia of the eyes is shown beside the figure of the fly. Study of these brings out the fact that the cell size is correlated directly with the chromosome quantity contained within them. Dobzhansky's extensive study of this same question utilizing the cell size of the wing arrives at the same conclusion for the secondary non-disjunctional forms.

The recent republication of Boveri's hypothesis of the cause of cancer as due to abnormal chromosome numbers within the cancer cells and Metcalf's revival of this hypothesis in his writings makes a consideration of these results from that view-point significant, since within the cells of the forms here described there is chromosome reduplication and chromosome unbalance. In outline Boveri's suggestion is based on the following reasoning: By fertilization of sea-urchin eggs with sperm in various ways abnormal chromatin forms could be produced. The cell size of these forms bore a correlation to the amount of chromatin contained in their nucleus. Mitoses were frequently abnormal. The number of chromosome combinations was very great, rivaling the number of types of tumors. Amitoses took place. The chromosome complex, incompatible in number, kind, or both, clearly caused these abnormal structural forms. As a parallel to this, cancer cells are frequently associated with enlarged cell size brought about by nuclear fusion. These cells may form irregular spindles and eventually pass into amitotic division. On this parallel Boveri based his suggestion. In a more general perspective, the case

appears even stronger, for as Wilson points out the limitations of growth and cell-division, while they may be controlled by a variety of agents, such as available food and hormones, are in the last analysis governed by heredity. This is obvious from the fact that specific mean size of organisms is hereditary. Histological analysis has shown that it is the number of cells rather than their size which governs an organism's mass. Mendelian analysis has shown that this size is determined by the genes within the chromosomes. Or speaking more broadly, the limits of cell-division and, therefore, growth are determined by the chromosome constitution of the cells. The mechanism controlling the amount of cell-division is therefore present in the cell itself and should it be released might well give the uncontrolled cell multiplication so impressive in tumor formation.

The facts deduced from this investigation are of particular significance to this hypothesis of Boveri. In this particular stock fertilized eggs are produced which in turn give rise to individuals which have 3 sex-chromosomes and 3 of each autosome group. While these individuals show a slightly larger cell size and body mass than the normal females of 2 sex-chromosomes and 2 of each autosome group, they are otherwise typically female in their organs, no noticeable abnormalities appearing. From this, the conclusion appears justified that the mere quantitative multiplication of the chromatin does not necessarily cause abnormal growths. The other type of abnormal eggs contains 3 sex-chromosomes, two of the X-type and one Y-chromosome, and 3 of each autosome. These individuals differ from both the normal male complex (one sex-chromosome of the X-type and one of the Y-type and 2 of each of the autosomes) and the normal female complex (2 sex-chromosomes of the X-type and 2 of each autosome group). Such adults are thus different in both the quantity and balance of the chromatin from the normal individuals. These individuals present abnormalities which are of wide range and clearly marked but they seem to result in a restraint in growth rather than a complete lack of its control. In fact, the abnormal organs formed and their peculiar association, *i.e.*, ovary and testis in the same animal or spermathecae associated with testes, are in general much reduced in size. In the germ cells, cell division and the normal maturation process appear hampered, but few cells going through the complete processes to egg

or sperm formation, sterility due to this excessive cell restraint apparently always resulting. These results, therefore, tend to negative Boveri's suggestion if they are accepted as comparable material on which a test of his hypothesis could be made. These organisms do have a characteristic which might conceivably produce a difference in reaction from that Boveri postulates, namely, they presumably have all their cells with the given abnormal chromosome complex. The result might well be different if only certain of the cells were abnormal in their chromosomes and growing perhaps at the expense of the rest. Such cases, known in *Drosophila* as gynandromorph and mosaic flies, have areas which differ from the rest of the body areas in the number of their chromosomes without the production of abnormal growth. The results agree with Boveri's in showing that the chromosome complex markedly influences growth.

SUMMARY

This paper presents an analysis of the type parents and atypical forms found within a special stock of *Drosophila melanogaster*. The anatomical changes observed in the abnormal individuals of this stock consist very largely in over or under growth of the organs, especially those of the reproductive system, bristles and wings. They involve alterations in the testes, ovaries, paragonia, vas deferens, ejaculatory sac, oviduct, spermathecae, parovaria, ventral receptacle and uterus, as well as the external genitalia, sex-combs, clasper plates and anal plates. Fertility is reduced. The duration of life is shortened. Bristles are reduced and wings cut. These forms appear sporadically within a certain family or its near relatives. The ratio of the abnormal individuals to normal varies anywhere from 1 in 26 to 1 in 100. Profound alterations are found within the cells of these abnormal individuals. The chromosomes are increased in number, 12 chromosomes instead of 8 being ordinarily found in the cells. The anatomical changes within the abnormal flies are shown to be associated with the alterations of this chromosome number. The agent which brings about these changes is a third chromosome recessive gene.

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